VOLUME 35 DECEMBER 1957 NUMBER 12

Canadian Journal of Biochemistry and Physiology

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY

(Formerly Canadian Journal of Medical Sciences)

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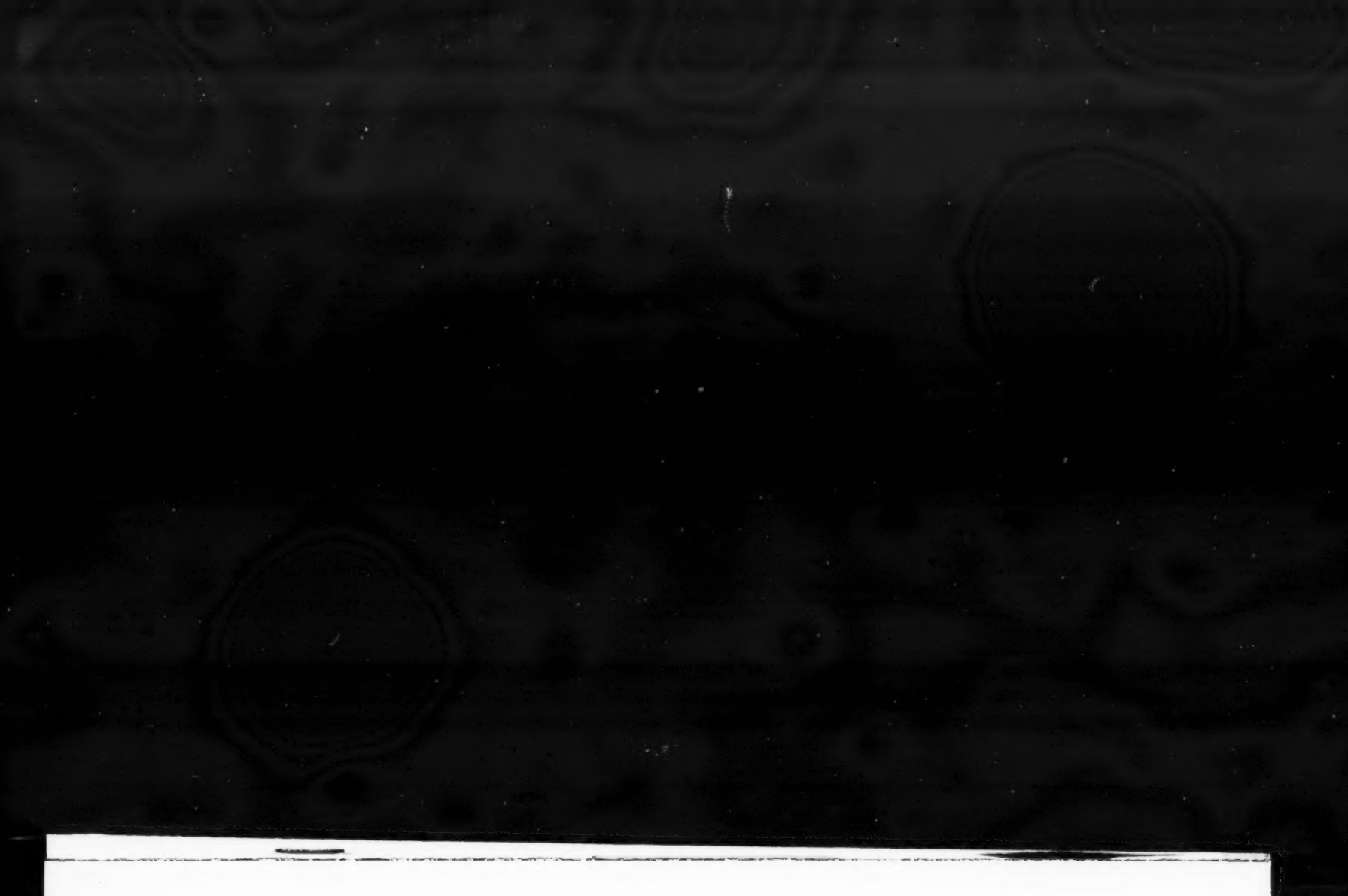

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Canadian Journal of Biochemistry and Physiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 35 DECEMBER 1957 NUMBER 12

THE DETECTION OF ESTROGENIC ACTIVITY IN TISSUES OF STEERS WHICH HAVE BEEN FED DIETHYLSTILBESTROL[1]

G. S. WIBERG AND N. R. STEPHENSON

Abstract

The immature female rat was used to detect estrogenic residues in the tissues of cattle fed a ration containing diethylstilbestrol. The criterion of response to the estrogen was the increase in uterine weight which resulted from the ingestion of the beef tissues. The weanling rat was twice as sensitive as the ovariectomized mouse to oral diethylstilbestrol. Significant levels of the estrogen were found in lean meat, liver, and kidney 24 hours after the removal of the diethylstilbestrol from the steers' diet, but such residues could not be detected when this period was extended to 48 hours.

Introduction

A number of investigators have used the increase in uterine weight of mice to measure estrogenic residues in the tissues of beef cattle treated with diethylstilbestrol (4, 5, 6, 7, 8, 9, 10). With ovariectomized mice, Stob, Andrews, and Zarrow (6) obtained a significant response in the uterine weight with 0.0055 mcg. diethylstilbestrol per g. of diet, while Umberger (10), using immature mice, could detect 0.002 mcg. diethylstilbestrol per g. of diet. Recently, Umberger and co-workers (11, 12) reported that the type of tissue in the diet affected the uterine weight of test animals as well as the slopes of the dose–response curves. This indicated that the estimation of estrogenic residues by the interpolation of the uterine weights on a single standard curve is not a valid procedure.

Gossett, Smith, and Downing (4) reported that three factors affected the level of residual estrogen in treated steers, viz. (i) the length of time between the final estrogen treatment and slaughter of the animals, (ii) the amount of diethylstilbestrol ingested per day, and (iii) its preferential storage in certain tissues.

Ellis, Allen, and Whiting (3) fed extracted tissues from diethylstilbestrol-treated lambs to weanling rats and employed the uterine response to test for completeness of extraction. Dorfman and Dorfman (2) used the increase in weight in the immature rat uterus as the criterion of response for the quantitative bio-assay of estrogens employing both the oral and subcutaneous

[1] Manuscript received July 8, 1957.
Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada. Presented at the 20th Annual Meeting of the Canadian Physiological Society, October 18–20, 1956.

routes of administration. These findings suggested that the weanling rat might be a suitable test animal for the bio-assay of estrogenic residues by the feeding technique.

In the present study, the uterine response of the immature rat to oral estrogen was compared to that of the ovariectomized mouse. Experiments were designed to investigate the effect of beef tissues per se on the uterine weight of the weanling rat and on the slope of the dose–response curve. In addition, beef samples were examined for estrogenic activity 24 and 48 hours after the removal of diethylstilbestrol from the diet.

Experimental

The tissue samples were obtained from four groups of steers under test at Macdonald College, McGill University. Each group consisted of three animals: Group I served as a control group while Group II was fed a supplement containing diethylstilbestrol. The ration fed to Group III contained streptomycin and that given to Group IV included both streptomycin and diethylstilbestrol. Each steer in Groups II and IV ingested 10 mg. diethylstilbestrol per day for a total of 110 days. The time interval between the withdrawal of the estrogen and the slaughter of the steers was 48 hours for Group II and 24 hours for Group IV. In the present study, it is assumed that streptomycin had no effect on the absorption and retention of diethylstilbestrol by the cattle.

Samples of lean meat (neck trim), liver, kidney, and perirenal depot fat were taken from each steer at the time of slaughter. These tissues were kept in a frozen state until they were examined. After thawing, the connective tissue and fat were removed from the lean meat, liver, and kidney. Each of these tissues was comminuted in a large Waring Blendor. An accurately weighed amount (usually 2 kg.) was dried in an oven with air circulation at 50–60° C. for 24 hours and reweighed. The dried tissue was passed through a meat grinder and mixed with 25% of its weight of ground laboratory chow (Master Fox cubes). The perirenal depot fat was minced with a meat grinder and mixed with sufficient chow to make a final beef fat concentration of 30%.

Laboratory chow was used throughout these experiments as a basal ration for the rats and mice. Diets were prepared in which known amounts of diethylstilbestrol were added to either the ground chow or to the dried tissue samples from control steers. These rations were used to establish the sensitivity of the method and to study the effect of the individual tissues on the uterine response to the estrogen. The small amount of kidney tissue available required the use of a composite sample.

Both female intact rats, 21–23 days old, and ovariectomized adult mice were employed in this study. The mice were castrated at 2–3 months of age and held for 30 days following the operation to permit full uterine atrophy (6).

The animals in groups of 10 were housed in cages, either singly or in pairs, and allowed tap water ad libitum. Food intake was restricted to 6 g. per animal per day for 8 days unless otherwise indicated. The animals were

sacrificed 24 hours after the last feeding. The uteri were excised, stripped of fat under a dissecting microscope, dried on filter paper, and weighed on a Roller Smith torsion balance. The results were expressed as mg. of uterus per 100 g. of body weight and represented the mean from 10 rats.

Results and Discussion

The relation between the age of the immature rat and the relative uterine weight was determined in groups of rats 21 to 41 days old. Fig. 1 shows that the relative uterine weight remained constant until the animals were 32 days old. Hence it may be assumed that any significant increase in the weight of the uterus during this period can be attributed to a uterotrophic agent, such as estrogen, in the diet. In subsequent tests, rats 21–23 days of age were employed in the tests and were sacrificed before 31 days of age.

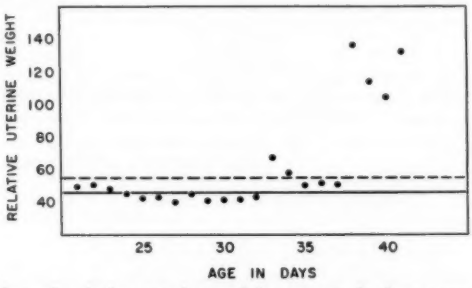

FIG. 1. Relation of relative uterine weight to age in immature rats. Solid line represents the mean uterine weight per 100 g. body weight in animals between 21 and 32 days old. Broken line represents least significant difference from this mean ($P < 0.05$).

The initial study of the uterine response to diethylstilbestrol was made with both immature rats and ovariectomized mice. Unmodified chow was fed to a control group while the other groups were given rations which contained diethylstilbestrol at levels of 0.005, 0.01, and 0.02 mcg. per g. of diet respectively. With immature rats, a comparison was made between ad libitum feeding (ca. 8–10 g. per day) and a restricted intake of 6 g. per day. Table I indicates that the weanling rat was sensitive to a diet containing 5 parts

TABLE I

UTERINE WEIGHT RESPONSE OF TEST ANIMALS TO GRADED LEVELS OF DIETHYLSTILBESTROL IN THE DIET

Diethylstilbestrol (mcg./g. of diet)	Uterine weight (mg.)/100 g. body weight		
	Immature rat, ad libitum feeding	Immature rat, 6 g. food/day	Ovariectomized mouse, ad libitum feeding
Nil (control)	46.3 ± 3.8*	49.0 ± 2.4*	53.5 ± 3.6*
0.005	70.3 ± 12.2	72.6 ± 4.1	52.5 ± 3.8
0.01	99.1 ± 4.0	89.5 ± 3.9	77.5 ± 5.5
0.02	170.6 ± 17.4	131.3 ± 6.4	107.8 ± 7.7

* Standard error of the mean.

per billion of diethylstilbestrol (0.005 mcg./g.) whereas the ovariectomized mouse required 10 parts per billion (0.01 mcg./g.) for a significant response. Table I also reveals that the restricted allowance did not affect the sensitivity of the test adversely but did reduce the standard error of the mean. Furthermore, with an intake of 6 g. per day, a reasonably accurate estimate of the food consumption was available in terms of the original sample.

The results obtained by feeding various tissues with known diethylstilbestrol content to intact immature rats are given in Table II. Lean meat, liver, and fat, without added estrogen (control), did not influence the uterine weight significantly. The sensitivity of the response seemed to be slightly reduced in the animals on a high fat diet. In contrast, Table III shows that the relative uterine weight was greater in the group given beef kidney than that in the rats receiving laboratory chow. Turner (9) has recorded a similar response to beef kidney in ovariectomized mice.

TABLE II

INFLUENCE OF THE COMPOSITION OF THE DIET ON THE UTERINE WEIGHT RESPONSE OF WEANLING RATS TO GRADED LEVELS OF DIETHYLSTILBESTROL

Diethylstilbestrol (mcg./g. diet)	Composition of diet (mg. uterus/100 g. body weight)			
	Laboratory chow	Lean meat	Liver	Fat
Nil (control)	49.0 ± 2.4*	48.9 ± 2.0*	43.6 ± 1.7*	47.1 ± 2.1*
0.005	72.6 ± 4.1	64.4 ± 3.7	55.2 ± 1.6	43.9 ± 1.5
0.01	89.5 ± 3.9	71.8 ± 3.3	59.2 ± 1.9	53.2 ± 1.9
0.02	131.3 ± 6.4	76.5 ± 2.7	82.1 ± 4.3	79.9 ± 3.3
0.03	—	86.0 ± 4.2	—	90.5 ± 6.0
0.04	—	—	154.7 ± 15.0	—

* Standard error of the mean.

Analyses of variance were made on the data reported in Table II. While the regression coefficient (*b*) was found to be significant, the deviation from regression was also significant. This suggested that the simple dose–response relation was not linear and attempts were made to find a suitable function which would produce a straight line. The following parameters were studied: log dose vs. response, dose vs. log response, log dose vs. log response, dose vs. square root of response, and dose vs. reciprocal of response. Although none of these functions produced a linear relation as determined by analysis of variance, graphically a straight line appeared to fit the data when the dose was plotted against the log mean response. Umberger (10) in a similar study with weanling mice found that a dose – log response relation was apparent with low levels of dietary diethylstilbestrol.

A possible reason for this failure to achieve statistically significant linearity might be attributed to the considerable spread in the initial body weight of the immature rats. In order to compensate for this variation in size of the test animals, the amount of diethylstilbestrol ingested was calculated per unit of body weight. This correction revealed that a highly significant

linear relation existed between the dosage of diethylstilbestrol per g. of body weight and the log of the relative uterine weight. The slopes of the regression lines for liver (0.0518) and depot fat (0.0580) did not differ significantly from that for laboratory chow (0.0823). The dose–response lines which resulted from feeding either laboratory chow, liver, or perirenal depot fat rations containing added diethylstilbestrol were found to be a linear function of the dose and converged at zero dosage. Therefore it should be possible to apply a slope–ratio assay for measuring the amount of estrogen in an unknown sample. Such an assay would necessitate the addition of diethylstilbestrol at two or more dose levels. The experimental design and calculations employed in this type of assay are described by Bliss (1).

In contrast the regression coefficient for lean meat (0.0259) was significantly different from that for laboratory chow. Extrapolation to zero dosage yielded a value (60.9) which was considerably higher than that encountered in untreated animals fed a diet containing a similar proportion of lean meat from untreated steers.

TABLE III

ESTROGENIC ACTIVITY OF TISSUES FROM STEERS WHICH HAD RECEIVED DIETARY DIETHYLSTILBESTROL

Steer No.	Lean meat	Liver	Depot fat	Kidney*
	(mg. of uterus/100 g. body weight)			
	(*a*) Group I—control			
1	47.4 ± 2.5†	48.7 ± 1.3†	40.8 ± 1.5†	
2	45.5 ± 1.7	42.0 ± 1.4	41.7 ± 1.3	
3	44.4 ± 2.4	42.1 ± 2.1	44.5 ± 1.6	
	45.6 ± 1.6	44.3 ± 1.3	42.3 ± 1.1	58.4 ± 2.5
	(*b*) Group II—diethylstilbestrol withdrawn 48 hours			
4	45.6 ± 2.3	42.3 ± 1.5	41.0 ± 1.4	
5	47.9 ± 1.6	46.7 ± 1.6	42.1 ± 1.2	
6	42.8 ± 2.1	46.2 ± 1.7	42.8 ± 1.3	
	45.4 ± 1.4	45.0 ± 1.4	42.6 ± 1.1	59.4 ± 3.7
	(*c*) Group III—streptomycin			
7	49.8 ± 2.8	48.3 ± 1.4	44.8 ± 1.0	
8	47.9 ± 1.4	43.9 ± 1.4	50.1 ± 1.4	
9	46.1 ± 1.9	50.4 ± 2.2	53.7 ± 1.7	
	47.9 ± 1.7	47.5 ± 1.6	49.5 ± 1.2	53.3 ± 2.1
	(*d*) Group IV—streptomycin + diethylstilbestrol withdrawn 24 hours			
10	56.4 ± 3.8	55.5 ± 3.2	42.5 ± 1.3	
11	60.5 ± 4.8	92.1 ± 12.0	44.3 ± 1.6	
12	63.0 ± 5.6	137.2 ± 12.2	51.3 ± 1.8	
	60.9 ± 3.7	94.9 ± 7.6	46.0 ± 1.5	163.7 ± 8.9

* Composite tissue sample.
† Standard error of the mean.

Table III illustrates the results obtained when tissues from treated steers were fed to immature rats. No estrogenic activity was found in any of the tissues of the steers which had been without diethylstilbestrol for 48 hours before slaughter. However, when this time interval was reduced to 24 hours, significant levels were found in the lean meat, and highly significant levels in the liver and kidney. Although the depot fat did not show any residual estrogen, it was not possible to feed as much of this tissue as the others. In terms of the original tissue, each rat on test consumed a total of 80–90 g. of liver, kidney, and lean meat, whereas only a little over 14 g. of fat was eaten during the same test period. Stob *et al.* (8) were able to demonstrate estrogenic activity in the perirenal depot fat of cattle given oral diethylstilbestrol by feeding a total of 25 g. of the fat to ovariectomized mice.

Although the relative uterine weight was higher in rats receiving kidney tissue as shown in Table III, precocious opening of the vagina, a manifestation of estrogenic activity, was observed only in those rats receiving the diet containing kidney tissue from the steers of Group IV.

The amount of estrogen in the different tissues of each animal in Group IV was variable. Steer 10 contained only traces in the lean meat and liver. Steers 11 and 12 had approximately the same level of estrogenic activity in the lean meat, but Steer 12 had a much higher amount of activity in the liver. Other investigators have used only composite samples from steers under test, and hence, have not reported this variability of tissue estrogen content in different steers given the same treatment.

Acknowledgments

The authors wish to thank Professors E. W. Crampton, James Hamilton, and G. E. Bradford of Macdonald College, McGill University, for providing the beef tissue samples used in this investigation. The staff of Canada Packers Ltd., Montreal and Hull, Que., aided considerably in the collection and delivery of the samples. The valuable assistance of Dr. W. P. McKinley and Mr. W. D. Snair of this laboratory is gratefully acknowledged.

References

1. Bliss, C. I. The statistics of bio-assay. Academic Press, Inc., New York. 1952. p. 560.
2. Dorfman, R. I. and Dorfman, A. S. Endocrinology **55**, 65 (1954).
3. Ellis, P. J., Allen, C. E., and Whiting, F. Can. J. Agr. Sci. **34**, 393 (1952).
4. Gossett, F. O., Smith, F. A., and Downing, J. F. Symposium on medicated feeds. Medical Encyclopedia Inc., New York. 1956. p. 97.
5. Preston, R., Cheng, E., Story, C. E., Homeyer, P., Pauls, J., and Burroughs, W. J. Animal Sci. **15**, 3 (1956).
6. Stob, M., Andrews, F. N., and Zarrow, M. X. Am. J. Vet. Research, **15**, 319 (1954).
7. Stob, M., Andrews, F. N., Zarrow, M. X., and Beeson, W. M. J. Animal Sci. **13**, 138 (1954).
8. Stob, M., Perry, T. W., Andrews, F. N., and Beeson, W. M. J. Animal Sci. **15**, 997 (1956).
9. Turner, C. W. J. Animal Sci. **15**, 13 (1956).
10. Umberger, E. J. Symposium on medicated feeds. Medical Encyclopedia Inc., New York. 1956. p. 28.
11. Umberger, E. J., Curtis, J. M., and Gass, G. H. Federation Proc. **15**, 1221 (1956).
12. Umberger, E. J., Gass, G. H., and Curtis, J. M. Federation Proc. **16**, 1525 (1957).

THE INTERRELATION OF THE GROWTH AND METABOLIC RESPONSES TO ANTERIOR PITUITARY GROWTH HORMONE ADMINISTRATION[1]

GEORGE H. BEATON, HANNAH Z. BANKY, AND AUDREY M. HAUFSCHILD

Abstract

Doses of growth hormone which were minimal with respect to body weight increase were sufficient to produce significant alterations in liver alanine – glutamic transaminase and arginase activities and blood urea and amino nitrogen levels. The biochemical effects of the hormone appeared coincident with the body weight increase. Female rats showed a more pronounced response to growth hormone than did male rats. This sex difference was evident with respect to all of the metabolic alterations observed. Although it is not possible to state whether the metabolic alterations are direct effects of the hormone, they do take an integral part in bringing about the over-all biological effect.

Introduction

It has been known for many years that suitably prepared extracts of the anterior pituitary gland, when injected into animals, could cause an increase in body weight. Fractionation of the crude extracts led to the isolation of growth hormone as the active constituent. It has been repeatedly demonstrated that administration of growth hormone to rats leads to an increased retention of protein and water and usually a loss of fat (6,7,17). The mechanism by which these changes are brought about is still unknown. There are many established metabolic alterations which follow the administration of the hormone (see Discussion). Some of the enzyme changes seem to be integral parts of the mechanism by which new protein is deposited. Other changes might be due to the nonspecific utilization of the protein moiety of the enzymes as a source of labile protein. This view has been put forward by Gaebler, particularly with reference to alanine – glutamic transaminase (15). One approach to this problem of the specificity of changes is to study the effects of small dosages of the hormone which are minimal with respect to the stimulation of body weight increase, and to study the sequence of alterations during hormone treatment.

Materials and Methods

Wistar strain rats from the Carworth Farms colony were employed. In each case, the animals were fed a 20% casein, 20% corn oil diet (5) for 2 weeks prior to the experiment. The animals were divided into groups equal with respect to size, sex distribution, and initial average body weight. The rats were housed in individual screen-bottomed cages in a room maintained at 75 $\pm$ 2° F. and provided with the 20% casein, 20% corn oil diet, and water

[1] Manuscript received June 26, 1957.
Contribution from the Department of Public Health Nutrition, University of Toronto, Toronto, Ontario. This work was supported by a grant from the National Research Council of Canada.

ad libitum throughout the experimental period. The growth hormone used in these studies was prepared by Connaught Medical Research Laboratories (lot 100) and was made available by the National Research Council.

In the first experiment, two groups of adult female rats having an average body weigh of 247 g. were employed in an attempt to find the minimal effective dose of growth hormone to produce body weight increases. In the treated group, the daily dose of growth hormone was increased stepwise from 200 μg. to 1000 μg. as shown in Fig. 1. The hormone was administered subcutaneously in 0.9% saline. Animals in the control group received comparable volumes of saline. The animals were killed on the 28th day of the experiment after having received the highest dose of the hormone for 7 days.

In the second experiment, the rats were divided into 7 groups of eight male and eight female rats each. The average body weights were: males, 291 g.; females, 213 g. One group, to be used as an initial control group, was fasted and killed immediately. The remaining groups were given daily subcutaneous injections of either saline or 2 mg. growth hormone per day. Saline- and growth hormone-treated groups were killed after 2, 5, and 13 days of injections.

In every case the animals were killed by stunning and decapitation following an 18 hour fast. No injections were given on the day of killing. Blood was collected from the neck in heparinized tubes. Livers were removed and prepared immediately for enzyme analyses. Blood assays were carried out by the following procedures: amino nitrogen, Frame *et al.* (14) as modified by Russell (25); urea, Archibald (1); sugar, Nelson (24); packed cell volume by the standard procedure. Alanine – glutamic transaminase activities of liver homogenates were determined by the method of Tonhazy *et al.* (26) as modified by Caldwell and McHenry (11); activities are expressed as the μl.

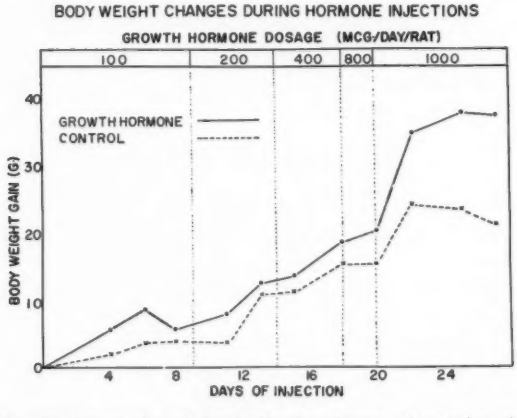

FIG. 1. Determination of the minimal effective dosage of anterior pituitary growth hormone in intact female rats. No significant difference in body weight was produced until the dose reached 1000 μg. per rat per day.

of pyruvate-CO_2 formed per mg. wet tissue per hour. Liver arginase activities were measured by the method of Van Slyke and Archibald (27) as adapted to liver tissue by Liener and Schultze (19); results are expressed as the micromoles of urea formed per 100 mg. wet tissue per minute. Statistical analyses were carried out by means of the "t"-test.

Results

The actual amounts of growth hormone administered in the first experiment and the body weight responses are shown in Fig. 1. The final body weight difference was significant at the 1% level ($t = 3.32$). From these data it can be concluded that the minimal effective dosage of growth hormone for body weight response in these rats was between 800 and 1000 μg. per day. However, since the dose was gradually increased and the rats had been receiving the hormone for 3 weeks before the 1 mg. level was reached, the threshold for response may have been altered. It is possible that a smaller dose given for a longer period of time would have caused a body weight increase. However, the promptness of response to the 1000 μg. dose gives the impression that it was minimal. The hormone had little or no effect on food intake. During the last week of the experiment, the average food intakes in g. per rat per day were as follows: growth hormone, 11.9; saline, 11.4. The body weight gains were not a result of an increased food intake.

TABLE I

BIOCHEMICAL ALTERATIONS PRODUCED BY MINIMAL DOSES OF GROWTH HORMONE
(Mean ± standard error)

Assay	Growth hormone treated	Saline treated	"t"	Level of significance
Blood				
Packed cell volume	44.6 ± 0.7	45.4 ± 0.3	1.04	—
Urea	20.7 ± 1.8	26.7 ± 2.2	2.12	5%
Amino nitrogen	10.3 ± 0.3	8.2 ± 0.3	5.27	1%
Sugar	109 ± 5.7	99 ± 3.1	1.54	—
Liver				
Alanine – glutamic transaminase	44.6 ± 3.2	64.7 ± 4.7	3.57	1%
Arginase	39.5 ± 1.5	46.5 ± 2.4	2.44	3%

The data shown in Table I indicate that the normal metabolic alterations previously observed with higher doses of growth hormone (6,7,9,10) are still evident with this minimal level of administration. The alterations included lowered blood urea, elevated blood amino nitrogen, and decreased liver alanine – glutamic transaminase and arginase activities.

In the second experiment, employing a higher level of growth hormone, body weight gains started at day 0 and steadily increased during the study. At the end of the experiment, the body weight gain of the growth hormone treated rats was significantly greater than that of the controls in both males

and females (significant at the 3% and 1% levels respectively). During the study, the hormone had very little effect on the food intake of the females (saline, 11.1; growth hormone, 11.6 g./rat/day), but it had a marked effect in the males (saline, 14.7; growth hormone, 17.3 g./rat/day). This increased food intake in the treated male rats was reflected by an increased deposition of fat; female rats showed very little change in body fat. The data on carcass composition are not reported in detail since the responses did not achieve statistical significance.

The biochemical alterations accompanying this treatment are shown in Table II. No changes in packed cell volume and liver arginase activity following hormone administration were noted, hence they have been omitted from the table. Liver arginase was significantly lower in female rats than in males at all times. A significant sex difference was also noted with respect to blood urea and amino nitrogen levels in control rats. Sex differences were also seen in the response to growth hormone. A statistically significant lowering of blood urea was seen in the females within 2 days; in the males, significance was not achieved at any time. The changes in blood amino nitrogen and transaminase activity were also more marked in the females than in the males. All of these changes began early in the experiment and progressively became more pronounced. The alterations coincided with the increase in body weight.

TABLE II

THE TIME OF APPEARANCE OF BIOCHEMICAL ALTERATIONS
(Mean ± standard error)

Group and time	Saline			Growth hormone		
	Blood urea, mg.%	Blood amino nitrogen, mg.%	Liver alanine-glutamic transaminase	Blood urea, mg.%	Blood amino nitrogen, mg.%	Liver alanine-glutamic transaminase
A. Males						
2 days	15.6 ± 1.3	11.9 ± 0.8	42 ± 4	14.1 ± 0.8	10.8 ± 0.5	42 ± 3
5 days	15.7 ± 1.1	11.9 ± 0.3	45 ± 3	14.5 ± 0.5	12.7 ± 0.2	32 ± 3*
13 days	16.8 ± 1.2	11.5 ± 0.2	39 ± 3	14.7 ± 1.0	12.0 ± 0.2	30 ± 2
B. Females						
2 days	19.8 ± 2.0	9.7 ± 0.2	47 ± 4	13.6 ± 1.2*	9.3 ± 0.3	41 ± 3
5 days	19.4 ± 0.6	10.1 ± 0.2	50 ± 3	15.8 ± 1.1*	12.2 ± 0.2*	24 ± 3*
13 days	24.3 ± 1.8	10.3 ± 0.3	43 ± 4	17.2 ± 1.7*	11.6 ± 0.1*	33 ± 3*

* The difference between means for this group and the comparable saline-treated group is statistically significant at the 1 or 2% level.

Discussion

Other workers have reported that growth hormone lowers blood amino nitrogen (23). Reports from our laboratory have described an elevation of blood amino acids following hormone treatment (6,7,10). This discrepancy may be explained by differences in the duration of treatment. In the present experiment it would appear that there is an initial lowering of amino nitrogen which is still evident after 2 days, followed by a significant elevation of the level. This might be due to a rapid incorporation of amino acids into tissue protein with a consequent lowering of blood amino acids followed by an increased production of available amino acids.

The marked sex difference in the response of rats to growth hormone is noteworthy. By means of pronounced enzyme changes, the female rat is able to increase body weight and retain protein without an increase in food intake. A definite increase in food intake in the male rat permits a gain in body weight with less marked alterations in enzyme activities than in the female. Deuel (13) has recently reviewed the subject of metabolic sex differences in man and in animals. He concluded that females exhibited a greater capacity to utilize body stores of fat and carbohydrate than did males. In our laboratory, female rats have consistently shown a more rapid and greater response to growth hormone than have male animals. The administration of oestrogen and progesterone to intact female rats increased the response to growth hormone (10). Bartlett was able to increase the response of female dogs to growth hormone by giving testosterone (2). A very definite influence is exerted by the sex hormones, whether endogenous or exogenous, on the metabolic effects of growth hormone.

A great deal of interest has been centered on the role of enzyme changes in the control of metabolism following growth hormone administration. A few of the liver enzyme activities that have been reported to be altered in intact rats are as follows: alanine – glutamic and aspartic – glutamic transaminases (6,7,9,10), arginase (6,9), D-amino acid oxidase (10), urea formation (9), fatty acid oxidase and esterase (18), and ketone body formation (12,18,20). Many changes have also been reported in hypophysectomized rats given growth hormone: kidney glutaminase (3), liver and kidney L-glutamic acid dehydrogenase (16), liver alanine – glutamic transaminase (10,28), and kidney, liver, and tibia alkaline phosphatase (21,22). It is impossible at this time to state definitely whether some of the enzyme changes are causative factors in the alterations in metabolism accompanying growth hormone treatment, or results of the changes in metabolism.

Gaebler (15) has dealt with this problem in a recent review article. He considered alkaline phosphatase in tibia, L-glutamic acid dehydrogenase and alanine – glutamic transaminase in liver, and aspartic – glutamic transaminase in muscle. It was felt that there was insufficient evidence as yet to postulate that the enzymes could be thought of as the primary pathway of growth hormone action. Many of the changes seemed to be of a secondary nature. The possibility that some enzymes are used up as a source of labile protein was also considered, with particular reference to alanine – glutamic transaminase.

An examination in our laboratory of the effects of a variety of experimental conditions on alanine – glutamic transaminase activity (8) implied that it was related to the direction of protein metabolism and did not represent a labile protein store. It has been demonstrated (9) that single injections of growth hormone can produce changes in liver alanine – glutamic transaminase and arginase activities and in the rate of urea formation by liver slices within 3 hours. These alterations clearly precede any changes in body weight and are not a reflection of a general withdrawal of protein from

the liver. On the other hand, attempts to produce *in vitro* effects of growth hormone on transaminase (4) and glutaminase (3) were unsuccessful, suggesting that the hormone does not act directly on the enzymes themselves.

When the effects of growth hormone are studied chronologically, it is apparent that changes in the activity of several enzymes are coincident with an increase in body weight, an increase which is mainly due to an increased retention of protein and associated water. The augmented storage of protein is made possible, even without an increased ingestion of protein, by metabolic alterations. It is likely that such metabolic alterations can be accomplished only by changes in enzyme activity. It has been shown in our laboratory that even if rats are maintained on low protein diets, the enzymes are not more markedly reduced by growth hormone treatment than if ample dietary protein is available. Evidence available at present does not support the idea that decreases in some enzymes occur because the protein moieties are used as a source of protein for tissue formation.

References

1. ARCHIBALD, R. M. J. Biol. Chem. **157**, 507 (1945).
2. BARTLETT, P. D. Endocrinology, **52**, 272 (1953).
3. BARTLETT, P. D. and GAEBLER, O. H. J. Biol. Chem. **181**, 523 (1949).
4. BARTLETT, P. D. and GLYNN, M. J. Biol. Chem. **187**, 253 (1950); **187**, 261 (1950).
5. BEATON, G. H. Arch. Biochem. Biophys. **67**, 1 (1957).
6. BEATON, G. H. Endocrinology, **59**, 356 (1956).
7. BEATON, G. H. and CURRY, D. M. Endocrinology, **58**, 797 (1956).
8. BEATON, G. H., CURRY, D. M., and VEEN, M. J. Arch. Biochem. Biophys. **70**, 288 (1957).
9. BEATON, G. H., OZAWA, G., BEATON, J. R., and MCHENRY, E. W. Proc. Soc. Exptl. Biol. Med. **83**, 781 (1953).
10. BEATON, G. H., RYU, M. H., and MCHENRY, E. W. Endocrinology, **57**, 748 (1955).
11. CALDWELL, E. F. and MCHENRY, E. W. Arch. Biochem. Biophys. **45**, 97 (1953).
12. CAMPBELL, J. and DAVIDSON, I. W. F. J. Biol. Chem. **189**, 35 (1951).
13. DEUEL, H. J., JR. Intern. Z. Vitaminforsch. **26**, 352 (1956).
14. FRAME, E. G., RUSSELL, J. A., and WILHELMI, A. E. J. Biol. Chem. **149**, 255 (1943).
15. GAEBLER, O. H. The hypophyseal growth hormone, nature and actions. Blakiston Division, McGraw-Hill Book Co., New York. 1955. Chap. 22, p. 383.
16. GAEBLER, O. H. and MATHIES, J. C. Endocrinology, **51**, 469 (1952).
17. GREENBAUM, A. L. Biochem. J. **54**, 400 (1953).
18. GREENBAUM, A. L. and MCLEAN, P. Biochem. J. **54**, 413 (1953).
19. LIENER, I. E. and SCHULTZE, M. O. J. Biol. Chem. **187**, 743 (1950).
20. LOTSPEICH, W. D. and PETERSEN, V. P. Am. J. Physiol. **176**, 232 (1954).
21. MATHIES, J. C. and GAEBLER, O. H. Endocrinology, **45**, 129 (1949).
22. MATHIES, J. C., GAEBLER, O. H., and PALM, L. Endocrinology, **45**, 480 (1949).
23. MILMAN, A. E. and RUSSELL, J. A. Endocrinology, **47**, 114 (1950).
24. NELSON, N. J. Biol. Chem. **153**, 375 (1944).
25. RUSSELL, J. A. J. Biol. Chem. **156**, 467 (1944).
26. TONHAZY, N. E., WHITE, N. G., and UMBREIT, W. W. Arch. Biochem. **28**, 36 (1950).
27. VAN SLYKE, D. D. and ARCHIBALD, R. M. J. Biol. Chem. **165**, 293 (1946).
28. ZUCHLEWSKI, A. C. and GAEBLER, O. H. Arch. Biochem. Biophys. **66**, 463 (1957).

A DEVICE TO FACILITATE THE TUBE-FEEDING OF SMALL ANIMALS[1]

R. J. G. Gillespie and C. C. Lucas

Abstract

A device is described that simplifies the tube-feeding of rats.

Introduction

Catheters and cannulae have been used for fifty years to tube-feed mice and rats (5, 2). Small animals usually resist attempts to pass a stomach-tube. Various types of gags have been proposed (1, 4, 3) to facilitate the operation, but in our hands none of these has been found to be entirely satisfactory. Sometimes the animals become so frantic that injury is caused to mouth, throat, and internal organs. The excellent method of holding a rat for the passing of a stomach-tube described by Shay and Gruenstein (6) is easily used on smaller rats, but did not eliminate struggling and danger to the operator when attempted with very large rats.

The device to be described is simple in design and easy to use.* Rats do not object to it, probably because there is no struggle to force the mouth open, and hence it is unnecessary to alarm them further by holding them tightly. Thus the 'stress' to which the animal is subjected is minimized. There is nothing for the rat to push against with its paws and the natural tendency to push with its snout merely improves the positioning of the device. A fine rubber catheter, or better, a plastic infant-feeding tube,† slips easily through the hollow central metal guide-tube, which directs the flexible tubing over the tongue and down the gullet and prevents the rat from biting the soft tubing. One person can conduct the operation with ease.

Construction

Two views of the tube-feeding aid are shown in Fig. 1 and the device in use is pictured in Fig. 2. The outer ring, which fits over the rat's snout, is made by cutting a short piece (approximately 0.25 in. (0.6 cm.)) from streamlined copper or brass tubing (so-called 1 in. (2.54 cm.) I.D.) The central guide-tube that directs the flexible stomach-tube to the back of the mouth is also made from copper or brass tubing (1 in. long, so-called 1/8 in. (0.32 cm.) I.D., 3/16 in. (0.48 cm.) O.D.). It is flared slightly at the outer end to make easier the introduction of the flexible plastic or rubber tubing. This piece of metal tubing is supported by an upcurved piece of heavy copper

[1]Manuscript received July 3, 1957.
Contribution from Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario.

*The units are commercially available from James H. Wilson Limited, 88 Adelaide St. W., Toronto, Ontario.

†A satisfactory plastic infant-feeding tube is model K-31, Pharmaseal Laboratories, Glendale 1, California.

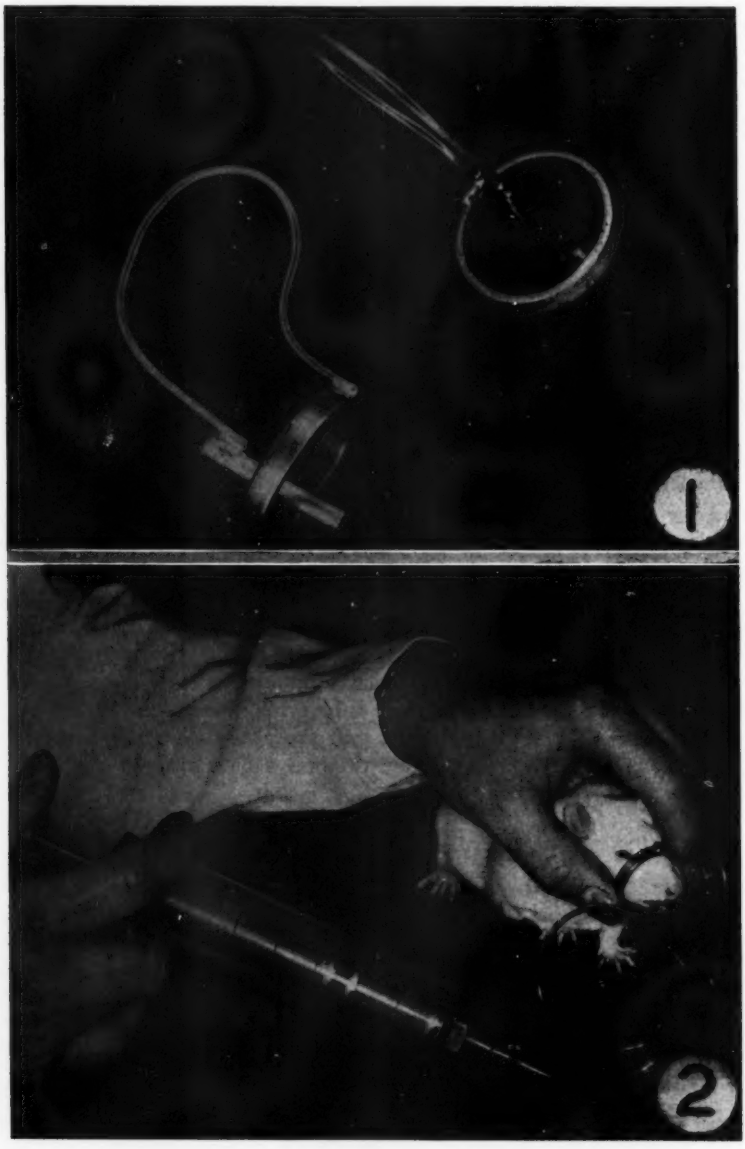

Fig. 1. Photographic views of the unit devised to simplify the forced feeding of rats by stomach-tube.

FIG. 2. Photograph showing operator force feeding a rat using the device pictured above and described in the text.

wire (No. 14 standard wire gauge, 3.75 in. long (9.5 cm.)) which is soldered to it and to the outer ring. The wire is bent in an arc to go above and in front of the rat's snout as shown in Fig. 2, and then back around to support the guide-tube in a horizontal position passing through the lower third of the vertical diameter of the outer ring.

Procedure

The rat is placed on a flat surface with the operator's left hand placed over its back as shown in Fig. 2. The device, held in the right hand by the curved piece of copper wire, is gently introduced by putting the metal guide-tube in the side of the mouth (to one side of the incisors) and gradually centering it as the outer ring is slipped in place around the snout. Now that danger of being bitten has been eliminated the ring is taken between thumb and forefinger of the left hand and is held snugly against the face of the rat. It is important to keep the supporting wire vertically above the snout as the animal is then unable to reach it with its paws. Some slight bending of the wire may be necessary to ensure comfortable adjustment of the metal guide-tube for rats of different sizes. When the device is in position, the rat is prevented from backing up by the operator's left hand and it cannot advance because of the ring held by the operator over its snout. Using the right hand, the feeding tube, previously attached to a syringe lying on the table, is now easily slipped through the guide-tube, down the oesophagus into the stomach. To indicate when the tip of the feeding tube is in the stomach, it is advisable to place a mark on the tubing at a point opposite the outer end of the guide-tube when the tubing is in the proper position.

Although we have not used the automatic volume dispenser for tube-feeding rats described by Talalay and Takano (7), it is our belief that use of such an apparatus along with the device here described would make the oral administration of foods or drugs (in suspension or solution) to rats of any size an easy routine procedure.

Acknowledgments

This unit was developed while we were working on a project supported by a grant from the Nutrition Foundation, Inc.

References

1. Burn, J. H. Biological standardization. Oxford University Press, London. 1937. pp. 71, 72.
2. Ferrill, H. W. J. Lab. Clin. Med. **28**, 1624 (1943).
3. Lehr, D. J. Lab. Clin. Med. **30**, 977 (1945).
4. Machella, T. E. and Griffith, J. Q., Jr. The rat in laboratory investigation. *Edited by* J. Q. Griffith, Jr., and E. J. Farris. J. B. Lippincott Co., Philadelphia. 1942. pp. 164, 165.
5. Marks, L. H. J. Exptl. Med. **10**, 204 (1908).
6. Shay, H. and Gruenstein, M. J. Lab. Clin. Med. **31**, 1384 (1946).
7. Talalay, P. and Takano, G. M. V. J. Lab. Clin. Med. **40**, 486 (1952).

THE IDENTIFICATION OF A NINHYDRIN-POSITIVE URINARY COMPONENT RECENTLY REPORTED IN HYPOPHOSPHATASIA[1]

DONALD FRASER[2] AND C. C. LUCAS

Abstract

In 1955, the occurrence of a hitherto unreported ninhydrin-positive substance in the urine and plasma of infants suffering from hypophosphatasia was described. Evidence published at that time and subsequently suggested that the material might be phosphorylethanolamine, but the isolation and unequivocal identification of the substance has not been reported.

The present paper describes the isolation of a small amount of the previously unidentified compound from the urine of the heterozygous father of a seriously affected infant. The melting point, mixed melting point, X-ray diffraction pattern, infrared absorption spectrum, and N : P ratio establish the identity of the substance to be phosphorylethanolamine. Proof that this compound occurs in hypophosphatasia provides a logical basis for further study of the metabolic importance of this substance.

Hypophosphatasia is a rare clinical entity belonging to that group of genetically determined diseases known as "the inborn errors of metabolism". The syndrome is characterized by abnormal mineralization of bone, and diminished serum and tissue alkaline phosphatase activity (1). A third, and probably characteristic, feature was added in 1955 when McCance *et al.* (2) and Fraser *et al.* (3), working independently, discovered the presence of a hitherto unreported ninhydrin-positive substance in the urine and plasma of infants with the disease. Paper chromatographic techniques indicated that the material might be phosphorylethanolamine (aminoethyl phosphoric ester) and data obtained subsequently have tended to support the suggestion (4, 5). However, none of the recorded findings can be considered to have established the identity of the material with adequate certainty. The isolation of the substance in crystalline form from urine, and its identification, are reported in the present paper.

The urinary concentration of the material is low in individuals with hypophosphatasia, and practical difficulties in the collection of sufficiently large amounts of urine from small infants have precluded its isolation from the urine of our patients. However, hypophosphatasia appears to be transmitted on an autosomal recessive basis (1), and, although parents of affected children do not have the characteristic skeletal lesions, most have been found to exhibit low serum alkaline phosphatase activities, and to excrete what appears by chromatographic and other criteria (6) to be the same unidentified urinary component. The isolation to be described was made from the urine of the heterozygous father of a seriously affected infant.

[1]Manuscript received July 16, 1957.
Contribution from The Research Institute, The Hospital for Sick Children, Toronto, Ontario, and from the Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada.

[2]John and Mary R. Markle Scholar in Medical Science, Department of Paediatrics, University of Toronto, Toronto, Canada.

Experimental

1. *Isolation and Crystallization of the Urinary Component*

The unidentified substance (Compound X) was isolated from 13.3 liters of urine of the father of T. L. and R. L. (Cases 9 and 19, quoted in Reference 1). The urine was preserved with thymol and stored at 4° C. for several weeks prior to extraction.

The first steps followed the general procedure outlined by Westall (7). Cation- and anion-exchange columns separated the unknown compound from a large proportion of undesired urinary components; it was further purified via the barium salt and crystallized from aqueous ethanol. Filter paper partition chromatography (8), which provided a rough measure of the degree of purification and of the yield attained at each step, was employed to follow the progress of the fractionation.

Preliminary removal of most of the urinary proteins, some of the inorganic salts, and other substances that interfere with ion-exchange chromatography was effected by concentrating the urine *in vacuo* to 1120 ml. in a rotary flash-evaporator* and adding three volumes of ethanol. A heavy, flocculent gray-brown precipitate that formed carried down some of the compound. The precipitate was washed with three 100 ml. portions of 80% ethanol. The clear, dark brown supernatant (plus the alcohol washings) was concentrated *in vacuo* to 930 ml. to remove the ethanol. Attempts to obtain further fractionation by the addition of ethanol failed.

Paper chromatographic analysis of the aqueous solution, Fraction A, indicated that it contained ninhydrin-positive substances in approximately the same relative concentrations as did the original urine. A weakly positive ninhydrin reaction was present in the position characteristic of Compound X. Glycine occurred in relatively high concentration, moderate amounts of alanine, serine, and taurine were present, and there were traces of aspartic and glutamic acids and other unidentified substances.

(*i*) *Fractionation by Cation-Exchange*

A cation-exchange column was used to attain the first major separation of amino acids and other ampholytes. Column CI (height 37 cm., diameter 4.5 cm.) constructed according to the descriptions of Partridge and Brimley (9), and of Westall (7), contained 260 g. (dry weight) of the synthetic cation-exchange resin, Permutit Q^R·† (40–60 mesh), the latter being employed in the hydrogen form. Fraction A, diluted to 26.6 liters with ion-free water, was applied to the top of Column CI at a rate of 200–300 ml. per hour. The effluent was collected in 250 ml. aliquots. Each aliquot was examined for pH, ninhydrin reaction, and sodium content (flame photometer).

After the passage of 250 ml. of liquid, the effluent became and remained strongly acidic. Coincident with the appearance of the inorganic acids, the ninhydrin reaction became positive and remained so thereafter. Compound

*Manufactured by Laboratory Glass Supply Company, New York 31, U.S.A.

†Permutit Q is a product of the Permutit Company, Birmingham, New Jersey, U.S.A., and was obtained from the Permutit Company of Canada, Ltd., Toronto, Ontario.

X and taurine were found to pass unimpeded through the column of Permutit Q, whereas a large proportion of all the other amino acids was retained by the column. The appearance of sodium in the effluent constituted a practical indication that the cation-exchange column had become exhausted.

Effluents 1–32 (total volume 9000 ml.), which were sodium-free, contained sufficient Compound X to give a weak ninhydrin reaction. Relatively large amounts of taurine were present, and small amounts of other amino acids were observed. Effluents 33–36, containing Compound X contaminated with sodium, were returned to the unprocessed portion of Fraction A.

Preliminary studies having shown that neither Compound X nor taurine was present in eluates of the cation-exchange resin obtained by displacement with 0.5 *N* ammonium hydroxide, the column was regenerated in the usual manner (7), with rejection of the substances that had been adsorbed (glycine, alanine, serine, histidine, glutamic and aspartic acids, and several other ninhydrin-positive components). In a manner similar to that described, the remaining 17 liters of Fraction A were processed in three portions through the regenerated column of Permutit Q.

The many sodium-free effluents from the cation-exchange column were combined to form Fraction B (32 liters). It contained high concentrations of inorganic acids, a moderate amount of urinary pigment, and small amounts of ninhydrin-positive materials. The latter consisted mainly of taurine, with relatively low concentrations of Compound X, glutamic acid, glycine, and unidentified amino acids. Undetermined amounts of uncharged components (urea, creatinine, and sugars) were present.

(*ii*) *Fractionation by Anion-Exchange*

A system of anion-exchange columns (7) was employed to achieve additional separation of the components in Fraction B. Five columns, AI (height 24 cm., diameter 5.3 cm.); AII (height 9.5 cm., diameter 2.8 cm.); AIII (height 7.0 cm., diameter 1.4 cm.); AIV (height 4.0 cm., diameter 0.7 cm.); AV (height 3.5 cm., diameter 0.5 cm.) contained, respectively 286 g., 31 g., 5.7 g., 0.8 g., and 0.4 g. (dry weight) of the synthetic polystyrene resin, Dowex 2-X8[R],*, the latter being employed in the hydroxyl form. Resin of 50–100 mesh was used in the three larger columns, and resin of 100–200 mesh in the two smaller ones. The columns were connected in series of decreasing size, an appropriate combination of the above units being selected for each fractionation. Care was taken to use carbon dioxide-free solutions during the regeneration and operation of the anion-exchange columns.

Because of the high concentration of inorganic anions in Fraction B with a relatively small amount of ninhydrin-positive material, an intentionally crude anion-exchange separation was carried out as a preliminary procedure. Fraction B, previously heated to approximately 60° C. under vacuum to remove dissolved carbon dioxide, was applied to Columns AI and AII, connected in series, at a rate of 500–1000 ml. per hour, and the effluent was

*Dowex 2, a product of the Dow Chemical Company, Midland, Michigan, was kindly donated by the Dow Chemical Company of Canada, Ltd., Toronto.

collected in 250 ml. aliquots. The first 10 liters of effluent (pH 5.6) were colorless and ninhydrin-negative, indicating that the resin had retained all the acid radicals, urinary pigments, and amino acid derivatives, while presumably the uncharged urinary components mentioned previously had passed through. After approximately one-third of Fraction B had been applied, yellow pigment appeared in the effluent, and a positive ninhydrin reaction was obtained, indicating that the capacity of the system had been exceeded. Application of Fraction B was therefore stopped, and the column was washed with ion-free water (1 liter per hour) until the effluent was again neutral and ninhydrin-negative. The washings were saved if ninhydrin-positive.

The ninhydrin-positive components that had been retained on the column were displaced with 0.1 *N* hydrochloric acid applied to Column AI at a rate of 750 ml. per hour. The eluate from Column AII was collected in 250 ml. aliquots. The ninhydrin test was carried out on 25 μl. of eluate that had been dried on filter paper to remove the interfering hydrochloric acid. After application of approximately 750 ml. of acid, the eluate became ninhydrin-positive and yellow in color, the pH gradually becoming acidic. When nearly 1 liter of eluate had been collected, the ninhydrin reaction became negative, and chloride ion appeared in the eluate. In a similar manner, the remaining 20 liters of Fraction B were fractionated in two portions on regenerated Columns AI and AII.

The many ninhydrin-positive eluates that resulted from the preliminary fractionation of Fraction B were subjected separately to paper chromatographic analysis. According to their contents of Compound X, the various eluates were pooled into four fractions, C1, C2, C3, and C4, the characteristics of which are shown in Table I.

In chromatograms of Fraction C2, relatively intense ninhydrin reactions were observed in the positions of Compound X and taurine. A moderate amount of glutamic acid was present, glycine occurred in low concentration, and the presence of several unidentified ninhydrin-positive components was noted.

Fraction C2, which contained the larger proportion of Compound X, was subjected to more careful anion-exchange separation. The fractionation system consisted of Columns AI, AII, AIII, and AIV, but the two smaller

TABLE I

CHARACTERISTICS OF FRACTIONS C1–C4

Fraction	Volume, ml.	pH	Cl	Amino acid–ninhydrin reaction*	Compound X–ninhydrin reaction†	Remarks
C1	2200	5	0	Trace	0	Colorless, clear
C2	2650	4	0	+++	++	Pale yellow, sl. fine white ppt.
C3	875	1	+	++	+	Moderately yellow, mod. fine white ppt.
C4	1250	1	+++	++	0	Moderately yellow, sl. fine white ppt.

*Carried out on the solution.
†Carried out on the chromatogram.

columns were not connected initially, since they tended to reduce the flow-rate through the system. Fraction C2 (volume 2650 ml.) was freed of dissolved carbon dioxide, and applied to Column AI at a rate of 500 ml. per hour. After the application had been completed, pigment bands occupied the upper fifth of the column. A small sample of colorless effluent withdrawn at this moment from the tube connecting Columns AI and AII had pH 5 and was ninhydrin-negative, i.e. Fraction C2 had not exceeded the capacity of Column AI.

After 1400 ml. of ion-free water had been passed through the system, 0.1 *N* hydrochloric acid was applied at a rate of 250 ml. per hour to displace the ninhydrin-positive components.

Periodic sampling of the effluent flowing from Column AI to AII demonstrated that the ninhydrin front coincided approximately with the foremost pigment band. Adsorption of ninhydrin-positive substances on Column AII was observed to cause the resin bed to *swell*, in contrast to the shrinkage noted when adsorbing chloride ions. By observing the position of the foremost pigment band and the behavior of the resin bed, it was possible to locate the ninhydrin-positive components at any stage of fractionation.

Column AIII was connected in series as soon as the ninhydrin-positive front reached Column AII, and Column AIV was connected when the front reached Column AIII. The rate of application of the hydrochloric acid was reduced to 50 ml. per hour at this stage. The eluate was collected in small aliquots (70 drops = 5.3 ml.) using an automatic "drop-count" fraction collector.*

The eluate became ninhydrin-positive after 4600 ml. of 0.1 *N* hydrochloric acid had been applied. The following 350 ml. of eluate (Fractions D1 to 66) were ninhydrin-positive, and contained varying intensities of urinary pigment which varied in color from yellow to pink-orange. Chloride ion appeared in the eluate after Fraction 58. Water-insoluble white or yellow precipitates appeared in some fractions in moderate amounts. They were not Compound X and were discarded.

The distribution of the ninhydrin-positive components of Fractions D1–66 is indicated in Fig. 1. Fractions D13–19, containing most of the Compound X, were pooled to form Fraction E (total volume 37.2 ml., pH 3). Considerable amounts of yellow and white amorphous precipitates were removed by centrifugation. The straw-colored supernatant was subjected to paper chromatographic analysis. An intense ninhydrin reaction was observed in the position characteristic of Compound X; moderately strong reactions were obtained for taurine and glutamic acid, and there were several unidentified weakly positive spots.

Fraction E, applied as a dilute solution, was refractionated on a small anion exchange system consisting of Columns AIII, AIV, and AV in series. The ninhydrin-positive substances were displaced slowly with 0.1 *N* hydrochloric acid as previously, and were collected in 11 aliquots (each 4.5 ml.).

*Manufactured by The Technicon Company, Chauncey, New York, U.S.A.

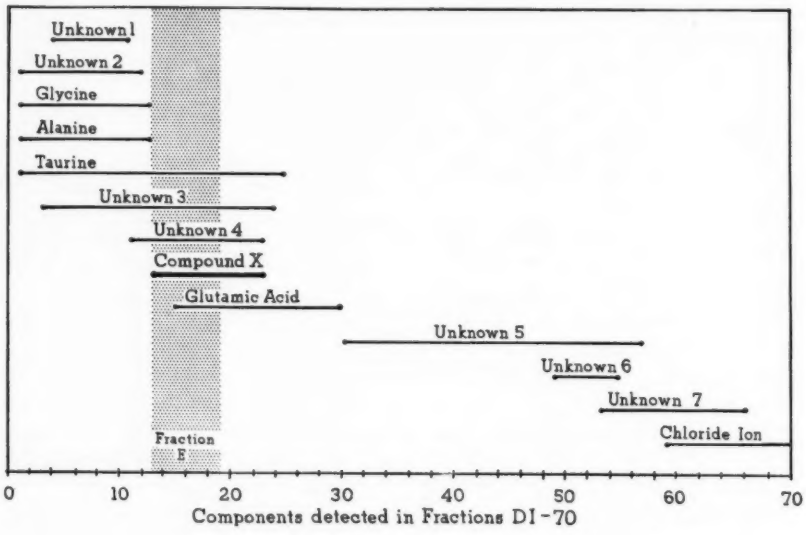

FIG. 1.

Compound X was found mainly in four of these that later were combined to form Fraction F.

Fraction F (volume 18.0 ml.) was straw-colored and contained a moderate amount of white precipitate which was removed. The major ninhydrin reaction occurred in the position of Compound X, but there were still four weakly positive spots, one of which was thought to be taurine. In retrospect, the authors question the effectiveness of this step in the fractionation.

(iii) Isolation of Compound X from Fraction F

Fraction F was evaporated to dryness in a stream of filtered air at approximately 60° C. The gummy residue (0.403 g.), which had a caramelized odor and appearance, was dissolved in 1.0 ml. of water. Attempts to precipitate Compound X by the addition of ethanol produced a dark brown alcohol-insoluble gum, most of which redissolved readily in a small volume (0.3 to 0.6 ml.) of water. Small amounts of water-insoluble, gray to white material remained behind at each repetition of the alcohol precipitation. Paper chromatograms demonstrated that Compound X occurred only in the water-soluble fraction. When no further water-insoluble material was eliminated by this procedure, the dark-brown, water-soluble supernatant was treated with an excess of barium hydroxide. Chromatographic analysis indicated that Compound X remained in the water-soluble fraction. The insoluble barium salts were removed by centrifugation. Precipitation of the water-soluble barium salts with various concentrations of ethanol (40–66%) resulted

in the formation of dark brown gummy precipitates containing Compound X. These were pooled, and barium was removed exactly by careful addition of dilute sulphuric acid. The solution was concentrated to a dark brown, slightly viscous sirup; this was placed under a small bell jar with a dish of ethanol. The latter diffused gradually into the aqueous layer. With scratching, a semicrystalline, dark brown granular mass was obtained (18.6 mg.). This was dissolved in 5 ml. of water and the solution was shaken with approximately 0.5 mg. of phosphorus-free activated charcoal.* The charcoal was removed by filtration.

The almost colorless solution was concentrated to a brown gum (8.2 mg.) and redissolved in 0.15 ml. of water. Alcohol was added from a microdropper to the light orange solution to give a final concentration of 40%, and the solution was allowed to cool slowly from 60° C. When the solution was allowed to stand overnight at 4° C., many small, well formed, slightly brown crystals appeared (Fig. 2). After the clear, light-brown supernatant was decanted, the crystals were washed twice with ice-cold 50% ethanol. The yield was 3.3 mg. (dried over silica gel).

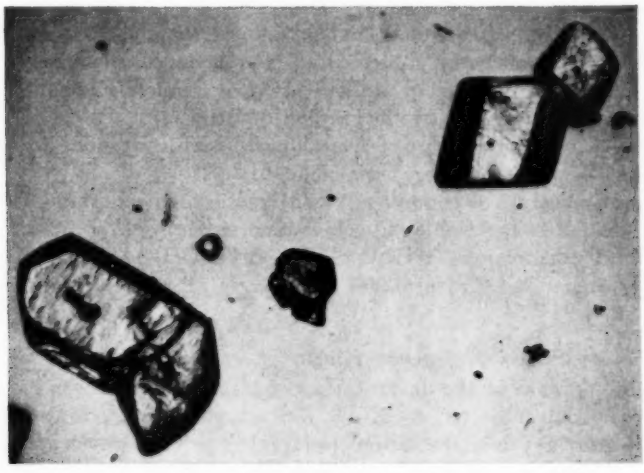

FIG. 2. Crystalline Compound X (phosphorylethanolamine).

2. Identification of Compound X

Many similarities between the unidentified material and phosphorylethanolamine had already been noted:

(1) The unknown substance was shown (4) to contain a phosphoric ester linkage by the technique of Hanes and Isherwood (10).

(2) The chromatographic partition coefficients of the unknown compound (in water-saturated phenol containing a trace of NH_3, $R_f = 0.33$, and in water-

*B.D.H. charcoal washed exhaustively with dilute hydrochloric acid, with alcohol, and with ether.

saturated 2,4,6-collidine-2,4-lutidine (1:1), $R_f = 0.097$) were not measureably different from corresponding data for synthetic phosphorylethanolamine.

(3) The unknown material behaved on cation- and anion-exchange columns in a manner similar to that of phosphorylethanolamine (4, 5).

(4) The movement of the unidentified material during paper electrophoresis coincided with that of the synthetic substance (5).

The isolation of only 3.3 mg. of crystalline material obviously limited elementary analysis and precluded further purification. However, the following determinations on the isolated crystals of Compound X confirmed the identity with synthetic phosphorylethanolamine.

(i) Melting Point

Crystals of Compound X melted by the hot-stage technique at 238–239° C. (corrected). This temperature was six degrees less than expected. (Pure phosphorylethanolamine* with a capillary melting point of 244–245° C. (11) was used to standardize the apparatus.) The mixed melting point was 240–241° C. (corrected).

(ii) Phosphorus and Nitrogen Determinations

Aliquots from an aqueous solution containing 1.166 mg. of crystalline Compound X in 5.00 ml. were subjected to microchemical analysis. Nitrogen was estimated in duplicate by nesslerization of a micro-Kjeldahl distillate. Phosphorus was measured by the method of Brun (12).

The mean nitrogen content of two aliquots, each containing 0.466 mg. of Compound X, was 0.0374 mg., equivalent to 8.02% N.

The mean phosphorus content of two aliquots, each containing 0.0466 mg. of Compound X, was 0.00806 mg., equivalent to 17.3% P.

Phosphorylethanolamine (NH_2-CH_2-CH_2-O-$PO(OH)_2$) requires N = 9.93%, P = 22.0%, molar N : P ratio = 1.00. Found, N : P ratio of Compound X = 1.03.

(iii) X-Ray Powder Diffraction Pattern

A minute amount of the unidentified crystalline material was ground to a fine powder and subjected to X-ray diffraction analysis using monochromatic Ni-filtered Cu K_α-radiation (λ 1.5418). The wavelengths of the prominent diffraction rings were measured, and the intensity of each was estimated visually on an arbitrary scale of 1 to 10. The pattern was in very close agreement with that reported by Baer and Stancer (11) for the range of wavelengths recorded (Table II).

(iv) Infrared Absorption Spectrum

Approximately 1 mg. of the unidentified material was examined by infrared spectroscopy, and the resultant spectrum was compared with that of synthetic phosphorylethanolamine. The crystalline materials were suspended in

*Crystals of pure synthetic phosphorylethanolamine were kindly provided by Dr. Erich Baer and Dr. H. Stancer of the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario.

TABLE II

X-RAY DIFFRACTION PATTERNS OF COMPOUND X AND SYNTHETIC PHOSPHORYLETHANOLAMINE

Compound X		Phosphorylethanolamine (11)	
Å	Intensity	Å	Intensity
		8.85	½
5.75	8	5.77	7
5.15	6	5.17	4
4.41	10	4.41	10
4.27	10	4.26	10
		3.88	8
3.83	10	3.84	8
		3.76	½
3.57	3	3.58	5
		3.48	½
		3.28	½
		2.91	1
		2.87	½
		2.71	3
		2.63	2
		2.47	3
		2.42	1
		2.22	3

nujol, and the spectra were recorded on a Perkin-Elmer double-beam infrared spectrophotometer (Model 21). The excellent correspondence of the two patterns is shown in Fig. 3.

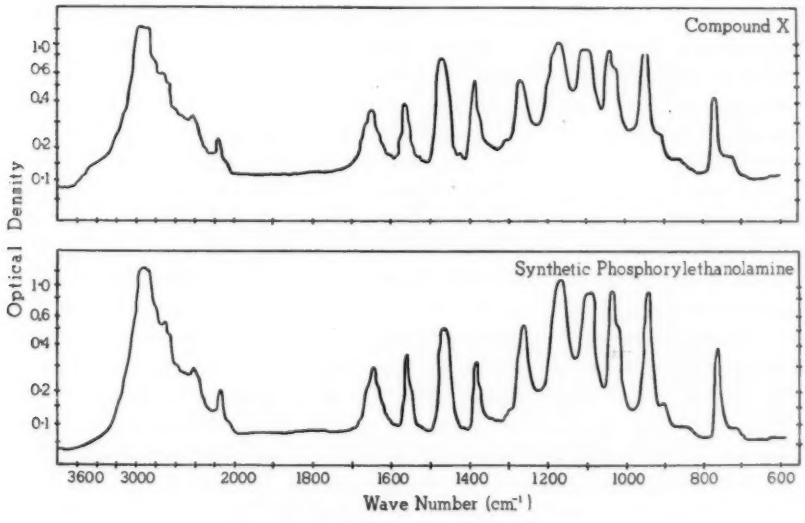

FIG. 3. Infrared absorption spectra.

The crystals isolated were admittedly somewhat impure as indicated by their slightly brownish coloration, low melting point, and low values for N and P. However, the evidence obtained from determination of the mixed melting point, the N : P ratio, the X-ray diffraction pattern, and the infrared absorption spectrum proves beyond any reasonable doubt that the urinary component under study is phosphorylethanolamine. The evidence provided by the so-called "finger-print" techniques of X-ray diffraction and infrared absorption is particularly convincing, since these latter methods are extremely specific, in many instances even in the presence of considerable amounts of contamination.

Discussion

In 1936, Outhouse discovered the presence of phosphorylethanolamine in animal material (13). By fractionation with alcohol of the water soluble barium salts extracted with acid from tissues, he was able to isolate sufficient material from bovine tumor to establish its chemical identity. Although Outhouse (14) believed that the substance was peculiar to malignant tissue, Colowick and Cori (15) subsequently obtained phosphorylethanolamine from the intestine of normal rabbits and pigs by fractionation of the uranium salts with barium hydroxide. Stone (16) also described, in canine cerebral tissue, a less well characterized substance that he suggested might be phosphorylethanolamine.

Since the advent of chromatographic techniques, many workers have reported indications of the presence of phosphorylethanolamine in mammalian tissues (both normal and neoplastic) (17–26), in avian tissues (17), and in bacteria (27). More recently, its occurrence has been suggested in humans (1, 2, 3, 19, 28) in certain disease states. Although the evidence of the foregoing authors for suggesting the presence of phosphorylethanolamine has sometimes been strong, it is not justified to accept without qualification any identification based only upon the behavior of the substance on paper or ion-exchange chromatograms. An example of such a fallacy is illustrated by the recent demonstration that a substance in turtle muscle with a mobility identical with that of phosphorylethanolamine in phenol–lutidine chromatograms was, in fact, the O-phosphodiester of L-serine and ethanolamine (23).

Recent studies of the metabolic disturbances in hypophosphatasia have regularly demonstrated in urine the occurrence of a substance with many of the characteristics of phosphorylethanolamine. The same procedures have failed to demonstrate such a material in the urine of children and adults with normal serum phosphatase activity. Since there is no report in the literature of the rigorous identification of the above substance in human material, it was considered important to establish its identity unequivocally. Our evidence proves beyond any reasonable doubt that the previously inadequately defined urinary component is phosphorylethanolamine, a finding which is corroborated by the recent independent studies of Cusworth (29).

Proof that this compound occurs in hypophosphatasia provides a logical basis for further study of the metabolic importance of this substance.

Note added in proof—Following submission of the manuscript, Mrs. Ruth Jahn (The Research Institute, The Hospital for Sick Children, Toronto), successfully isolated phosphorylethanolamine from the urine of a child with hypophosphatasia (T.L. Case 9, Reference 1). Identification was confirmed by infrared absorption spectroscopy.

Acknowledgments

The authors wish to thank Professor B. F. Crocker, Department of Biochemistry, University of Toronto, for his assistance in carrying out the melting-point measurements. The microchemical determinations were kindly made by Dr. Otokar Sirek and Mrs. Ruth Jahn, The Research Institute, The Hospital for Sick Children, Toronto. Much helpful advice and assistance was given throughout the study by Professor Erich Baer and Dr. D. Buchnea, Banting and Best Department of Medical Research, University of Toronto.

In particular, the authors wish to thank Dr. R. Norman Jones, Division of Pure Chemistry, National Research Council, Ottawa, who carried out the infrared spectroscopy and Professor G. F. Wright, Department of Chemistry, University of Toronto, who made the X-ray diffraction pattern. Without their assistance, the unequivocal identification of the urinary component could not have been made.

References

1. Fraser, D. Am. J. Med. **22**, 730 (1957).
2. McCance, R. A., Morrison, A. B., and Dent, C. E. Lancet, **1**, 131 (1955).
3. Fraser, D., Yendt, E. R., and Christie, F. H. E. Lancet, **1**, 286 (1955).
4. Fraser, D. and Yendt, E. R. A.M.A. J. Diseases Children, **90**, 552 (1955).
5. Dent, C. E. Discussion *quoted by* Engfeldt, B. and Zetterström, R. Ciba Foundation Symposium, Bone structure and metabolism. J. and A. Churchill, Ltd., London. 1956. p. 266.
6. Fraser, D. Unpublished.
7. Westall, R. G. Biochem. J. **52**, 638 (1952).
8. Dent, C. E. Biochem. J. **43**, 169 (1948).
9. Partridge, S. M. and Brimley, R. C. Biochem. J. **51**, 628 (1951).
10. Hanes, C. S. and Isherwood, F. A. Nature, **164**, 1107 (1949).
11. Baer, E. and Stancer, H. C. Can. J. Chem. **34**, 436 (1956).
12. Brun, G. Bibliotek Laeger, **131**, 203 (1939).
13. Outhouse, E. L. Biochem. J. **30**, 197 (1936).
14. Outhouse, E. L. Biochem. J. **31**, 1459 (1937).
15. Colowick, S. P. and Cori, C. F. Proc. Soc. Exptl. Biol. Med. **40**, 586 (1939).
16. Stone, W. E. J. Biol. Chem. **149**, 29 (1943).
17 Gordon, A. H. Nature, **162**, 778 (1948).
18. Roberts, E. and Frankel, S. Cancer Research, **9**, 645 (1949).
19. Awapara, J., Landua, A. J., and Fuerst, R. J. Biol. Chem. **183**, 545 (1950).
20. Astrup, T., Carlström, G., and Stage, A. Acta Physiol. Scand. **24**, 202 (1951).
21. Ansell, G. B. and Dawson, R. M. C. Biochem. J. **50**, 241 (1951).
22. Campbell, P. N. and Work, T. S. Biochem. J. **50**, 449 (1952).
23. Roberts, E. and Lowe, I. P. J. Biol. Chem. **211**, 1 (1954).
24. Tallan, H. H., Moore, S., and Stein, W. H. J. Biol. Chem. **211**, 927 (1954).
25. Smith, L. C. and Rossi, F. M. Proc. Soc. Exptl. Biol. Med. **87**, 643 (1954).
26. Awapara, J. Texas Repts. Biol. and Med. **13**, 1 (1955).
27. Work, E. Biochim. et Biophys. Acta, **3**, 401 (1949).
28. Fisher, O. D. and Neill, D. W. Lancet, **1**, 334 (1955).
29. Cusworth, D. C. Personal communication. 1956.

BIOCHEMICAL STUDIES ON CHLORPROMAZINE

1. THE EFFECT OF CHLORPROMAZINE ON RESPIRATORY ACTIVITY OF ISOLATED RAT BRAIN CORTEX[1]

O. LINDAN, J. H. QUASTEL, AND S. SVED

Abstract

Chlorpromazine exerts a progressive inhibitory activity (at 0.3 – 0.6 m*M*) on the respiration of brain cortex in presence of either glucose, fructose, pyruvate, or L-glutamate. A similar progressive inhibition occurs with other phenothiazine derivatives such as methylene blue and phenergan. However, chlorpromazine does not inhibit oxygen uptake in the presence of succinate. Potassium-stimulated respiration is highly sensitive to chlorpromazine, as it is markedly diminished by 0.2 m*M* concentration of the drug, a concentration which does not affect the unstimulated respiration. The increased inhibition of potassium-stimulated respiration is only clearly seen during the early part of the experiment.

Chlorpromazine is bound by tissue constituents. At a constant concentration of chlorpromazine (0.6 m*M*), its inhibitory effect on cortical respiration may be abolished by markedly increasing the amount of tissue present. The inhibitory effect of chlorpromazine may be diminished by addition of plasma proteins ($\alpha\beta$-globulin) or by addition of heated homogenized brain, liver, or kidney. No binding occurs with polyglutamic acid, ribonucleic, and deoxyribonucleic acids, but binding does occur with certain acid dyes such as trypan red. Trypan red may be used to immobilize free chlorpromazine. When the latter drug is absorbed, however, by the nervous tissue, the addition of trypan red has no effect on the metabolic inhibitions brought about by the absorbed chlorpromazine.

It is concluded that chlorpromazine resembles a large variety of narcotics and anaesthetics in its marked inhibitory effects on potassium-stimulated respiration of the brain. Its action, *in vitro*, however, differs from that of the narcotics in bringing about progressive, apparently irreversible, inhibitions and in its high binding power with tissue proteins. Such apparently irreversible inhibition is consistent with the conclusion that the drug, after combination with the tissue, gradually diffuses into the cell bringing about metabolic inhibitions.

Introduction

The results of recent work (7,8) have shown that a variety of barbiturates and local anaesthetics have the property of depressing, at low concentrations, the potassium-stimulated respiration of brain cortex slices *in vitro*. The respiratory activities of unstimulated brain cortex slices in glucose media are also affected, as is now well known, but the percentage inhibitions are small at the low concentrations of the drugs that may bring about narcosis or anaesthesia. In the presence of concentrations of potassium ions, approximately those present in the interior of the neurone, the respiration of the nerve cell *in vitro* is increased to a value approaching that found *in vivo*, as long as glucose is also present. Apparently the effect of added potassium is to stimulate an aspect of nerve cell respiration that is concerned with complete

[1] Manuscript received July 16, 1957.
Contribution from the McGill – Montreal General Hospital Research Institute, 3619 University Street, Montreal, Quebec.
A preliminary statement concerning this work was given at the Research Conference on Psychopharmacology on March 28, 1955, McGill University, Montreal. It is reported, in part, in Progress in Neurobiology. Vol. 3. (*Edited by* S. R. Korey and J. I. Nurnberger.) Paul Hoeber & Co. 1957.

carbohydrate oxidation and which is highly sensitive to the action of barbiturates and local anaesthetics. With the advent of the tranquilizing drugs, it became of great interest to discover whether they behave *in vitro* in a manner similar to the barbiturates and local anaesthetics so far investigated.

The purpose of this communication is to describe results that have been obtained recently by us in an investigation of the effects of chlorpromazine and allied phenothiazine compounds on brain respiration *in vitro*.

Methods

Tissue Respiration

Standard Warburg manometric techniques have been used to measure the respiratory activities of brain cortex slices. Adult rats, weighing 150–220 g. each have been used for all the work. The animals were killed by decapitation and brain cortex slices were cut with a Stadie–Riggs slicer. They were cut parallel to the dorsal and lateral surface of the cerebral hemispheres, 80 mg. wet weight of the tissue per Warburg vessel usually being used except in two sets of experiments when 40, 80, and 160 mg. were used. The slices were allowed to respire in a phosphate–saline medium in an atmosphere of oxygen at 37°. Respired CO_2 was absorbed by potash placed in the center well.

The phosphate–saline medium used contained: NaCl, 128 m*M*; KCl, 5 m*M*; $CaCl_2$, 0.6 m*M*; $MgSO_4$, 1.3 m*M*; and sodium phosphate buffer of pH 7.4, 10 m*M*. This medium was used either without further additions, except for the tissue, for measuring "endogenous oxygen uptake" or in the presence of one of the following substrates: glucose, 10 m*M*; fructose, 10 m*M*; sodium pyruvate, 10 m*M*; sodium L-glutamate, 20 m*M*; sodium fumarate, 20 m*M*; sodium succinate, 30 m*M*. The total volume in the manometer flask was 3 ml.

Potassium-stimulated respiration of brain cortex slices was produced by adding 0.1 ml. KCl (3 *M*) from the side arm to the main Warburg vessel, thus obtaining a final concentration of 0.1 *M* KCl (7, 8).

The aqueous solution of the drugs under investigation (chlorpromazine, chlorpromazine sulphoxide, phenergan, methylene blue, chloretone, trypan red) was tipped from the side arms into the Warburg manometer main vessels 15 or 30 minutes after the first manometric reading.

Chlorpromazine Estimation

Estimations of chlorpromazine content in the media were carried out by a modified method of Durost and Pascal (3). The media were treated first with two volumes ethanol and then centrifuged. To the supernatant, which was immersed in an ice bath, an equal volume of cold concentrated H_2SO_4 containing 2% formalin was added very slowly in order to avoid charring of the organic matter. The color was left to develop at room temperature for 18 hours. Readings were taken in the Beckman spectrophotometer at 545 mμ and compared with those on a standard curve.

Results and Discussion

Effects of Chlorpromazine on Rates of Oxygen Uptake by Rat Brain Cortex Slices

When narcotics such as luminal or chloretone are added to brain cortex slices respiring in a glucose–phosphate–saline medium, the respiration falls within a few minutes to an equilibrium value which remains approximately constant for an experimental period of over one hour (10). A different phenomenon was observed with chlorpromazine. With this drug, an inhibition of respiration takes place which increases with time. Some typical results are shown in Table I. A concentration of 0.2 m*M* chlorpromazine gave no inhibition of respiration within the experimental error. Concentrations of 0.4 m*M* – 0.6 m*M* gave inhibitions of the order of 12–15% within the first half hour of the experiment and these inhibitions increased to 55–89% within the third half hour of the experiment. Such progressive inhibitions are not found with barbiturates or chloretone, when used at small concentrations, in the presence of brain cortex slices respiring in adequate phosphate–saline–glucose media (10). They are found, however, when ether is used *in vitro* as the narcotic (11). It is possible that the progressive inhibition of respiration shown by chlorpromazine with brain cortex slices is due to the gradual diffusion of the drug from sites on the cell surface, where it is first absorbed, into the cell interior.

TABLE I

EFFECTS OF CHLORPROMAZINE ON RESPIRATION OF RAT BRAIN CORTEX SLICES
(saline–phosphate–glucose medium)

Chlorpromazine, m*M*	Q_{O_2}		
	First 30 min. interval	Second 30 min. interval	Third 30 min. interval
0	11.8	11.4	10.6
0.2	11.4	11.0	10.4
0.3	12.0	11.0	8.0
0.4	10.4	9.2	4.8
0.5	10.2	6.6	2.4
0.6	10.0	3.8	1.2

This phenomenon of progressive inhibition of respiration of rat brain cortex slices was apparent not only with glucose as substrate but also when glucose was replaced by fructose, sodium pyruvate, sodium-L-glutamate, or sodium fumarate. It was also apparent with the "endogenous oxygen uptake" (no substrate added) but it was not apparent when sodium succinate was present as substrate. Typical results are given in Table II. In common with many narcotics investigated, chlorpromazine, when present even at relatively high concentrations, was unable to bring about a depression of respiration of brain cortex slices in the presence of succinate. It is evident that the succinic oxidase system is as resistant to the inhibitory effects of chlorpromazine as it is to the majority of narcotics. This lack of effect of

TABLE II

EFFECTS OF CHLORPROMAZINE (CP) ON RESPIRATION OF RAT BRAIN CORTEX SLICES IN SALINE–PHOSPHATE MEDIA WITH VARIOUS SUBSTRATES
(concentration of chlorpromazine 0.6 m*M*)

Substrate		Q_{O_2}					
		First 30 min. interval		Second 30 min. interval		Third 30 min. interval	
			With CP		With CP		With CP
Nil		4.8	3.4	3.6	0.8	2.0	0.4
Fructose	0.01 *M*	10.6	9.2	10.6	5.8	10.4	3.0
Sodium pyruvate	0.01 *M*	14.2	12.2	13.8	6.8	13.6	3.4
Sodium-L-glutamate	0.02 *M*	10.2	7.6	9.0	4.0	8.0	2.0
Sodium fumarate	0.02 *M*	4.6	3.6	3.4	1.8	1.8	0.6
Sodium succinate	0.03 *M*	13.0	12.8	11.0	11.4	9.2	10.2

TABLE III

COMPARISON OF THE EFFECTS OF SOME PHENOTHIAZINE DRUGS ON RESPIRATION OF RAT BRAIN CORTEX SLICES
(saline–phosphate–glucose medium)

Drug		Q_{O_2}		
		First 30 min. interval	Second 30 min. interval	Third 30 min. interval
Nil		11.8	11.4	10.6
Phenergan	0.3 m*M*	11.8	12.6	11.8
Phenergan	0.6 m*M*	11.2	6.4	1.6
Methylene blue	0.05 m*M*	15.6	15.0	12.0
Methylene blue	0.1 m*M*	14.2	12.2	8.6
Methylene blue	0.3 m*M*	15.4	7.0	2.8
Methylene blue	0.6 m*M*	14.2	9.2	3.2
Chlorpromazine–SO	6.0 m*M*	11.4	11.6	9.4
Chlorpromazine–SO	12.0 m*M*	12.6	10.2	4.4
Chlorpromazine–SO	24.0 m*M*	11.8	7.4	1.6

chlorpromazine on succinate respiration may be expected if the action of the drug is confined, like that of the barbiturates or chloretone, to the oxidation of carbohydrate or pyruvate through the citric acid cycle, the oxidation of endogenous DPNH* (4, 5) being particularly vulnerable.

The progressive type of inhibition found with chlorpromazine took place with other phenothiazine derivatives (Table III). Phenergan, initially less active as an inhibitor than chlorpromazine, gave an increasing rate of inhibition with brain tissue and at 0.6 m*M* ultimately gave a large percentage inhibition of the respiratory rate of rat brain cortex. The phenomenon was shown also with methylene blue (0.1–0.6 m*M*), but here the effect was two-phased. There was an initial phase when the presence of the methylene blue caused an increased respiratory rate (probably due to the dye acting as a hydrogen carrier) followed by a phase in which there was a progressive increase of

*DPNH = reduced diphosphopyridine nucleotide.

inhibition. This two-phase action of methylene blue has been commented upon in earlier investigations (12). Chlorpromazine sulphoxide inhibited respiratory activity of rat brain cortex but only at relatively high concentrations (6–24 m*M*).

The results with chlorpromazine presented on Tables I–III are similar in some respects to those reported elsewhere (1, 2, 6, 12).

Effects of Chlorpromazine on Potassium-Stimulated Respiration of Rat Brain Cortex Slices

In presence of added potassium ions, there is a stimulation of oxygen uptake of rat brain cortex slices respiring in a saline–phosphate–glucose medium, the stimulation being greatest during the first half hour of the experiment and gradually falling off during the remaining part of the experiment. Chlorpromazine exercised a decided inhibitory effect on the potassium stimulated respiration, this being only clearly seen during the early part of the experiment. A concentration of 0.2 m*M* chlorpromazine, which showed no inhibition of unstimulated brain cortex respiration, gave an inhibition of 18% of the stimulated respiratory rate. A concentration of 0.6 m*M* chlorpromazine inhibited unstimulated brain cortex respiration by about 15% and this value was increased to 41% with stimulated brain cortex respiration. Illustrative results are shown in Tables IV and V. Phenergan acted in a

TABLE IV

EFFECTS OF CHLORPROMAZINE ON POTASSIUM-STIMULATED RESPIRATION OF RAT BRAIN CORTEX SLICES IN A SALINE–PHOSPHATE–GLUCOSE MEDIUM; $K^+ = 0.1\ M$

Chlorpromazine, m*M*	Q_{O_2}		
	First 30 min. interval	Second 30 min. interval	Third 30 min. interval
0	18.8	16.8	12.6
0.2	15.4	15.0	12.0
0.4	15.8	14.0	13.0
0.6	11.0	7.4	3.4

TABLE V

COMPARISON OF PERCENTAGE INHIBITIONS BY CHLORPROMAZINE OF RAT BRAIN CORTEX RESPIRATION IN A SALINE–PHOSPHATE–GLUCOSE MEDIUM, WITH AND WITHOUT STIMULATION BY POTASSIUM IONS (0.1*M*)

Chlorpromazine, m*M*	Percentage inhibition			
	First 30 min. interval		Third 30 min. interval	
	Without added K^+	In presence of K^+	Without added K^+	In presence of added K^+
0.2	3	18	2	5
0.6	15	41	89	73

similar manner to chlorpromazine but was quantitatively not as effective. Methylene blue also exercised higher inhibitory effects on potassium-stimulated respiration of brain cortex than on the unstimulated respiration.

Additive Effects of Mixtures of Phenothiazine Derivatives and Narcotics on Brain Cortex Respiration

Chlorpromazine, at the concentration of 0.2 m*M* which was practically non-inhibitory with unstimulated brain cortex respiration, produced a marked inhibition if it was present together with another phenothiazine derivative (e.g. phenergan, chlorpromazine–sulphoxide, or methylene blue) at a concentration below that at which it exercised an inhibitory effect. This result makes it likely that the various phenothiazine derivatives are acting *in vitro* at the same, or a similar, site in the brain cell. Results in Table VI show the cumulative effect of combined subliminal doses of phenothiazine drugs.

TABLE VI

CUMULATIVE EFFECTS OF COMBINED SUBLIMINAL DOSES OF ANY TWO PHENOTHIAZINE DRUGS ON THE RATE OF RESPIRATION OF BRAIN CORTEX SLICES
(saline–phosphate–glucose medium)

Abbreviations: CP chlorpromazine; CP–SO chlorpromazine–sulphoxide; Phen phenergan; M-Bl methylene blue

Drugs				Q_{O_2} First 30 min. interval	Q_{O_2} Second 30 min. interval	Q_{O_2} Third 30 min. interval
Phen	0.3 m*M* +	CP	0.2 m*M*	11.8	5.8	2.0
Phen	0.3 m*M* +	CP	0.3 m*M*	11.4	4.0	1.0
Phen	0.3 m*M* +	CP–SO	6.0 m*M*	10.6	8.0	4.4
M-Bl	0.05 m*M* +	CP	0.2 m*M*	14.2	13.2	10.0
M-Bl	0.05 m*M* +	CP	0.3 m*M*	15.4	11.6	5.6
M-Bl	0.1 m*M* +	CP	0.2 m*M*	14.6	11.8	4.4
M-Bl	0.1 m*M* +	CP	0.3 m*M*	14.8	10.4	4.0
M-Bl	0.1 m*M* +	CP–SO	6.0 m*M*	13.2	10.4	5.6
CP–SO	6.0 m*M* +	CP	0.3 m*M*	10.6	6.8	3.8

TABLE VII

EFFECTS OF MIXTURES OF CHLORPROMAZINE AND CHLORETONE ON RESPIRATION OF RAT BRAIN CORTEX SLICES
(saline–phosphate–glucose medium)

Chlorpromazine, m*M*	Chloretone, m*M*	Percentage inhibition of Q_{O_2}: Without added K^+ First 30 min. interval	Without added K^+ Third 30 min. interval	With added K^+ (0.1 *M*) First 30 min. interval	With added K^+ (0.1 *M*) Third 30 min. interval
0	2.7	27	28	40	35
0.2	0	3	2	18	6
0.2	2.9	39	43	56	65
0.6	0	15	89	41	73
0.6	2.7	48	100	66	100

When chlorpromazine was mixed with a narcotic such as chloretone, the mixture showed additive effects on the brain cortex respiration. This occurred with both stimulated and unstimulated brain. Typical results are given in Table VII. These *in vitro* results may be considered as in accord with the clinical experience that preliminary administration of chlorpromazine increases sensitivity to subsequent administration of a narcotic.

Apparent Irreversibility of the Inhibition of Oxygen Uptake of Brain Cortex Slices Caused by Chlorpromazine

An immersion of brain cortex slices, even for a relatively short time, into a solution of chlorpromazine produced a permanent damage of the respiratory activity of the tissue. In this experiment the slices were incubated for 5, 15, and 30 minutes at 37° C. in a saline–phosphate–glucose medium containing 0.6 m*M* chlorpromazine. Subsequently the slices were taken out, washed, and transferred into new Warburg vessels containing the drug-free medium. The time which elapsed between the end of the incubation period and the beginning of the recording of the respiration in the new medium was about half an hour. Results in Table VIII show that in the treated slices there was a progressive decline of the rate of oxygen uptake, although they were in the drug-free medium. These results are consistent with the conclusion that chlorpromazine is absorbed by the tissue and gradually diffuses into the cells affecting metabolism there.

TABLE VIII

"IRREVERSIBILITY" OF THE RESPIRATORY INHIBITION OF BRAIN CORTEX SLICES CAUSED BY CHLORPROMAZINE (0.6 m*M*)

Time of preliminary incubation of brain slices in CP medium	Q_{O_2} in the drug free medium			
	First 30 min. interval	Second 30 min. interval	Third 30 min. interval	Fourth 30 min. interval
Control	11.6	11.0	10.6	9.8
5 min.	9.4	7.4	6.2	4.8
15 min.	5.6	3.2	2.0	1.4
30 min.	0	0	0	0

Affinity of Chlorpromazine to Proteins

When various amounts of rat brain cortex were used for the assay of respiratory activity by the Warburg manometric technique, it was found that doubling the amount of tissue, say from 80 mg. to 160 mg. wet weight, diminished the inhibitory action of a given concentration of chlorpromazine by more than half. Results illustrating this effect are shown in Table IX. These results are not usually obtained with narcotics such as the barbiturates or chloretone, where percentage inhibitory effects tend to be independent of the weight of tissue used. This is evidently not the case with chlorpromazine and the phenomenon is reminiscent of the toxic action of dyestuffs and other substances, in presence of proteins, on enzyme activities (9, 13, 14).

TABLE IX

EFFECTS OF INCREASING AMOUNTS OF RAT BRAIN CORTEX SLICES ON INHIBITION OF RESPIRATION BY CHLORPROMAZINE (0.6 mM)

Wet weight of tissue, mg.	Percentage inhibition of Q_{O_2}		
	First 30 min. interval	Second 30 min. interval	Third 30 min. interval
40	20	82	100
80	19	77	94
160	5	23	42

It is likely that the diminution of respiratory inhibition on increase of tissue weight is due to the absorption of chlorpromazine on some of the proteins of the tissue, leaving less of the drug available to attack the enzymes of the respiratory processes. This view is supported by the fact that the addition of heated tissue homogenates (brain, liver, kidney) to brain cortex slices greatly reduced the inhibitory effects of 0.6 mM chlorpromazine. Moreover, the addition of serum proteins to brain cortex slices brought substantial falls of the inhibitory effects of chlorpromazine. Typical results, given in Table X, show that the addition of a mixture of $\alpha\beta$-globulins secured protection of rat brain cortex slices against chlorpromazine, whereas neither serum albumin nor γ-globulin, at the same concentration, had any protective effects. These results lead to the conclusion that chlorpromazine has a high affinity for constituents of $\alpha\beta$-globulins, probably for the lipoproteins present.

In order to find more quantitative data about the relationship between the rate of respiratory inhibition and the drug–tissue ratio, the concentrations of the drug in the media were measured after incubation of 40, 80, and 160 mg. of brain cortex slices for periods of time between 15 and 120 minutes. The rate at which chlorpromazine was removed from the media (containing

TABLE X

EFFECTS OF SERUM PROTEINS ON INHIBITORY ACTION OF CHLORPROMAZINE (0.6 mM) ON RESPIRATION OF RAT BRAIN CORTEX SLICES (saline–phosphate–glucose medium)

Protein (4%) added	Q_{O_2} (in presence of chlorpromazine)		
	First 30 min. interval	Second 30 min. interval	Third 30 min. interval
γ-Globulin*	11.2	3.8	1.4
Albumin	12.4	8.0	2.4
$\alpha\beta$-Globulins†	11.8	11.4	9.4

* Cohn's fraction II.
† Cohn's fraction IV-4.

TABLE XI

THE ABSORPTION RATE OF CHLORPROMAZINE BY BRAIN CORTEX SLICES IN RELATION TO THE AMOUNT OF TISSUE PER WARBURG VESSEL AND THE OXYGEN UPTAKE

(Saline–phosphate–glucose medium with initial 0.6 m*M* concentration of chlorpromazine (CP))

Mg. (wet weight) brain cortex per vessel	Minutes	Total μl. O_2 per mg. dry cortex	CP	
			Concn. in medium, m*M*	γ absorbed by mg. dry brain cortex
40	0	0	0.60	0
	15	2.8	0.41	25
	30	4.9	0.32	38
	60	5.6	0.28	42
	120	5.6	0.23	49
80	0	0	0.60	0
	15	2.9	0.30	20
	30	5.3	0.22	25
	60	7.3	0.16	29
	120	8.0	0.14	31
160	0	0	0.60	0
	15	3.0	0.17	14
	30	5.9	0.14	15
	60	10.5	0.08	17
	120	16.2	0.07	18

initially 0.6 m*M* of the drug) by brain cortex slices is shown on Table XI. From these data it could be presumed that a marked inhibition of oxygen uptake occurred when the absorption of chlorpromazine by cortex slices reached the level of 25 γ per 1 mg. dry tissue.

Effects of Trypan Red on Chlorpromazine Inhibitions

The acid dyestuff, trypan red, readily forms with chlorpromazine a complex which has only little solubility in water. An equimolar mixture of trypan red (0.6 m*M*) and chlorpromazine (0.6 m*M*) was found to have no inhibitory effect on rat brain cortex respiration in a saline–phosphate–glucose medium indicating a relatively stable union between the two substances.

If a solution (0.6 m*M*) of trypan red, which exerted no toxicity on brain cortex respiration, was added to a medium containing brain cortex slices and chlorpromazine (0.6 m*M*), *after inhibition of respiration had been allowed to proceed for half an hour*, the inhibition continued to increase, as if the dyestuff had not been added. The result shows that the increasing inhibition must be due to the drug which had already been absorbed by the brain tissue and not to chlorpromazine free in solution. Typical results illustrating these facts are given in Table XII.

If, moreover, brain cortex slices were immersed in trypan red solution, and were then removed and washed, the highly stained brain cortex slices

TABLE XII

THE RATES OF OXYGEN UPTAKE BY RATS BRAIN CORTEX SLICES IN PRESENCE OF TRYPAN RED (TR, 0.6 m*M*) AND CHLORPROMAZINE (CP, 0.6 m*M*)

(saline–phosphate–glucose medium)

First half hour			Second half hour			Third half hour			
Compounds added		$\mu l.O_2$ per mg. dry tissue	Compounds added		$\mu l.O_2$ per mg. dry tissue	Compounds added		$\mu l.O_2$ per mg. dry tissue	Total $\mu l.O_2$ per mg. dry cortex
TR	CP	per 30 min.	TR	CP	per 30 min.	TR	CP	per 30 min.	per 1½ hours
−	−	6.9	−	−	6.6	−	−	6.2	19.7
+	−	6.8	+	−	6.6	+	−	6.3	19.7
−	+	5.8	−	+	2.2	−	+	0.7	8.7
+	+	6.6	+	+	6.5	+	+	5.3	18.4
+	−	6.5	+	+	6.2	+	+	5.1	17.8
−	+	5.0	+	+	2.0	+	+	1.3	8.3

respired at their normal rates in a glucose medium. The addition of chlorpromazine to such slices brought about its usual inhibitory effects. It is, therefore, evident that trypan red was absorbed at sites remote from those at which chlorpromazine was absorbed and which were not actively concerned with the brain cells' respiratory processes.

Other acid molecules, which might be expected to combine with chlorpromazine such as polyglutamic acid, ribonucleic acid, and deoxyribonucleic acids (presented as neutral sodium salts), were tried as possible protectors of brain cortex against chlorpromazine inhibitions but were found to be without effect.

Acknowledgments

Grateful acknowledgment is made to the Foundation's Fund for Research in Psychiatry (New Haven) and to the National Research Council of Canada for grants which made this work possible.

We are also indebted to Messrs. Poulenc, Ltd., Montreal, for their gift of phenothiazine drugs.

References

1. BALESTRIERI, A. and BERTI, T. Boll. soc. ital. biol. sper. **29**, 1669 (1953).
2. COURVOISIER, S., FOURNEL, J., DUCROT, R., KOLSKY, M., and KOETACHET, P. Arch. intern. pharmacodyamie, **92**, 305 (1953).
3. DUROST, P. and PASCAL, S. Ann. pharm. franç. **11**, 615 (1953).
4. ERNSTER, L., JALLING, O., LOW, H., and LINDBERG, O. Exptl. Cell Research, **9**, 124 (Suppl. 3) (1955).
5. ERNSTER, L., LOW, H., and LINDBERG, O. Acta Chem. Scand. **9**, 200 (1955).
6. FINKELSTEIN, M., SPENCER, J. A., and RIDGEWAY, E. R. Proc. Soc. Exptl. Biol. Med. **87**, 343 (1954).
7. GEDDES I. C. and QUASTEL, J. H. Anesthesiology, **17**, 666 (1956).
8. GHOSH, J. J. and QUASTEL, J. H. Nature, **174**, 28 (1954).
9. HOCHSTER, R. M. and QUASTEL, J. H. Ann. N.Y. Acad. Sci. **54**, 626 (1951).
10. JOWETT, M. and QUASTEL, J. H. Biochem. J. **31**, 565 (1937).
11. JOWETT, M. and QUASTEL, J. H. Biochem. J. **31**, 1101 (1937).
12. PERUZZO, L. and FORNI, R. B. Presse med. **61**, 1463 (1953).
13. QUASTEL, J. H. Biochem. J. **25**, 898 (1931); **25**, 1121 (1931).
14. QUASTEL, J. H. and WHEATLEY, A. H. M. Biochem. J. **25**, 629 (1931).

BIOCHEMICAL STUDIES ON CHLORPROMAZINE

2. EFFECTS OF CHLORPROMAZINE ON INCORPORATION INTO PROTEINS, AND BREAKDOWN OF GLYCINE-1-C^{14} BY ISOLATED RAT BRAIN CORTEX[1]

O. LINDAN,[2] J. H. QUASTEL, AND S. SVED

Abstract

Glycine is decomposed in rat brain cortex to yield carbon dioxide. This process, in which $C^{14}O_2$ is formed from glycine-1-C^{14}, is markedly stimulated by the presence of 10 m*M* glucose, the rate of production of $C^{14}O_2$ being increased at least threefold. The presence of succinate exercises a much smaller stimulation of $C^{14}O_2$ formation. The addition of KCl (0.1 *M*) or of 2,4-dinitrophenol (0.025 m*M*), whilst stimulating the rate of oxygen uptake, does not increase the rate of $C^{14}O_2$ formation from glycine-1-C^{14}. The addition of K^+ tends to diminish the rate. The process of glycine-1-C^{14} breakdown to $C^{14}O_2$ is almost insensitive to chlorpromazine, under the given experimental conditions, until relatively high concentrations (e.g. 0.6 m*M*) are used. The presence of chlorpromazine, however, brings about an inhibition of the rate of glycine-1-C^{14} incorporation into rat brain cortex proteins, an inhibition of 20% being recorded at a concentration of the drug (0.2 m*M*) that has little or no effect on the respiration of the brain or on the rate of breakdown of glycine-1-C^{14} into $C^{14}O_2$. Glycine incorporation into brain cortex proteins is a process relatively sensitive to chlorpromazine, the magnitude of inhibition being of the same order as that brought about by amytal at similar concentrations. It is suggested that chlorpromazine brings about its effects by an uncoupling of phosphorylation from oxidation in brain cortex slices.

Introduction

The purpose of this communication is to describe the effects of chlorpromazine on glycine-1-C^{14} incorporation into the proteins of rat brain tissue, and on glycine breakdown with formation of carbon dioxide, in the presence of rat brain cortex slices. We have shown in our preceding paper (6) that chlorpromazine exerts a progressive inhibitory effect on the respiration of rat brain cortex slices in the presence of glucose, fructose, pyruvate, or L-glutamate but not in the presence of succinate. The potassium-stimulated respiration is highly sensitive to chlorpromazine as it is markedly diminished by 0.2 m*M* concentration of the drug, a concentration which does not affect the unstimulated respiration. The increased effect of the drug on potassium stimulated respiration is only clearly seen, however, in the first half hour of the experiment. It was concluded that the drug, after combination with the tissue, gradually diffuses into the brain cells bringing about metabolic inhibitions.

Methods

Warburg Manometric Technique

Slices of adult rat cerebral cortex, weighing 70–90 mg. were allowed to respire in oxygen for 30 or 90 minutes at 37° in 3 ml. of a saline–phosphate medium containing: NaCl, 128 m*M*; KCl, 5 m*M*; $CaCl_2$, 0.6 m*M*; $MgSO_4$,

[1]Manuscript received July 16, 1957.
Contribution from the McGill – Montreal General Hospital Research Institute, 3619 University Street, Montreal, Quebec. Some of the facts given in this paper are reported in Progress in Neurobiology. Vol. 3. (*Edited by* S. R. Korey and J. I. Nurnberger) Paul Hoeber & Co. 1957.

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1.3 m*M*; Na phosphate buffer at pH 7.4, 10 m*M*. Substrates, when added to this medium, were glucose (10 m*M*) or sodium succinate (30 m*M*). Solutions of radioactive glycine were tipped from the side arms into each Warburg vessel after 30-minute equilibration at 37°. Stimulation of brain respiration, when required, was produced by adding either 0.1 ml. of 3 *M* KCl or 0.1 ml. of 7.5×10^{-4} *M* 2,4-dinitrophenol (DNP) from the side arm together with the glycine solution. Solutions (0.1 ml.) of chlorpromazine hydrochloride (CP) were also added simultaneously from the side arms giving final concentrations of the drug between 0.2 and 0.6 m*M*.

The center wells of the flasks contained 0.3 ml. of 0.5 *N* NaOH for absorption of CO_2. At the end of the incubation with glycine (30 or 90 minutes) 0.3 ml. 30% trichloracetic acid (TCA) was added to the main compartments of the flasks from the side arms to stop the reactions, but the shaking of the vessels in the bath was allowed to continue for a further 20 minutes to enable complete absorption of CO_2 to occur.

Radioactive Glycine Solution

A stock solution of radioactive glycine (glycine-1-C^{14}), 60 m*M*, was prepared by mixing appropriate quantities of radioactive and non-radioactive glycine solutions. The final concentration of glycine in the manometer vessels, after 0.1 ml. of the stock solution from the side tube was tipped into the main vessels, was 2.0 m*M*. The number of counts per minute of radioactive glycine per manometric vessel was 8.3×10^5.

Estimation of Glycine Incorporation into Brain Cortex Proteins

After the end of the manometric experiments the contents of the main compartments of the Warburg flasks were treated with 5 ml. 30% trichloracetic acid, the tissue was homogenized, and the suspension was centrifuged. The deposit was washed three times with 10 ml. 6% trichloracetic acid. The first washing was carried out at 90° for 10 minutes and the next two were carried out at room temperature. Subsequent washings were carried out with (*a*) 10 ml. 95% ethanol, (*b*) 10 ml. of a mixture of ethanol and ether (3:1, v/v) at 60° for 10 minutes, and (*c*) 10 ml. ether. Finally, the proteins were resuspended in 1.0 ml. of a mixture of chloroform and ether (4:1, v/v), transferred to aluminum plates, dried, weighed, and the protein radioactivities measured.

Estimation of CO_2 Formation from Glycine by Brain Cortex Slices

The contents of the center wells (NaOH and Na_2CO_3) were transferred into centrifuge tubes to which 0.1 ml. of 2 *M* NH_4Cl and 0.5 ml. of saturated solution (approx. 1.5 *M*) $BaCl_2$ were added. The precipitated $BaCO_3$ was washed three times with 5 ml. water and then 5 ml. acetone. After 1 hour drying at 110° the total $BaCO_3$ was weighed in the centrifuge tube. The $BaCO_3$ was then resuspended in approximately 1 ml. of acetone and transferred to the aluminum plates, weighed, and from the estimation of its radioactivity the total formation of radioactive CO_2 was calculated.

Results

Effects of Chlorpromazine on Glycine Metabolism in the Presence of Glucose

The results given in Table I show the inhibitory effects of chlorpromazine on glycine metabolism by rat brain cortex slices, during 30- and 90-minute periods, in a saline–phosphate medium containing glucose. With increase of concentration of the drug from 0.2 m*M* to 0.6 m*M* the incorporation of glycine into the brain proteins and the formation of CO_2 from glycine were progressively inhibited; at the same time a progressive inhibition of the respiration took place. The rate of glycine incorporation was affected to the greatest extent, the inhibition occurring earlier and being relatively greater than the inhibitions of glycine breakdown to carbon dioxide and of oxygen uptake. For example, the rates of oxygen uptake and formation of $C^{14}O_2$ during the first half hour of the experiment were not affected at all by the presence of 0.3 m*M* concentration of chlorpromazine, while the rate of incorporation of glycine was diminished by 30%.

TABLE I

EFFECTS OF CHLORPROMAZINE (CP) ON GLYCINE-1-C^{14} METABOLISM AND OXYGEN UPTAKE BY RAT BRAIN CORTEX SLICES IN A SALINE–PHOSPHATE–GLUCOSE MEDIUM

Duration of experiment, min.	CP, m*M*	Glycine incorporation per mg. protein		$C^{14}O_2$ formed per mg. dry tissue		Oxygen uptake per mg. dry tissue	
		c.p.m.	% inhibition	c.p.m.	% inhibition	μl. O_2	% inhibition
30	0	10.0	—	238	—	6.4	—
30	0.2	8.0	20	281	0	5.9	8
30	0.3	7.0	30	167	0	6.2	3
30	0.6	4.2	58	15	36	5.5	14
90	0	32.8	—	770	—	18.5	—
90	0.2	28.3	14	750	3	17.0	8
90	0.3	15.3	53	667	14	15.8	15
90	0.6	4.6	86	200	74	8.1	56

Effects of Chlorpromazine on Glycine Metabolism in the Absence of Glucose

The rates of glycine incorporation and breakdown to CO_2 by rat brain cortex slices in the absence of added substrate were approximately one-third of those obtained when glucose was present. The presence of succinate, although increasing markedly the rate of oxygen uptake by rat brain cortex slices, had no effect on glycine incorporation into the brain proteins. It brought about, however, an increased rate of glycine breakdown to CO_2, this however, being considerably less than that obtained in the presence of glucose. Typical results are shown in Table II.

The presence of chlorpromazine at 0.6 m*M* in the saline–phosphate medium, or in the saline–phosphate–succinate medium, brought about an inhibition of the rate of glycine incorporation amounting to 77% and of glycine breakdown to carbon dioxide of 60–75% (Table II). These figures compare well

TABLE II

EFFECTS OF CHLORPROMAZINE (CP) ON GLYCINE-1-C^{14} METABOLISM AND OXYGEN UPTAKE BY RAT BRAIN CORTEX SLICES
(Time = 90 min.)

Substrate (saline–phosphate medium)	CP, m*M*	Glycine incorporation per mg. protein		$C^{14}O_2$ formed per mg. dry tissue		Oxygen uptake per mg. dry tissue	
		c.p.m.	% inhibition	c.p.m.	% inhibition	μl. O_2	% inhibition
Nil	0	13.0	—	216	—	6.4	—
Nil	0.6	3.0	77	93	60	3.5	45
Sodium succinate	0	12.3	—	358	—	14.3	—
Sodium succinate	0.6	2.9	77	87	75	15.2	0

with the percentage inhibitions obtained by chlorpromazine when the rat brain cortex slices were incubated in the presence of glucose (Table I).

Effects of Chlorpromazine on Glycine Metabolism in the Presence of Excess Potassium Ions and of Dinitrophenol

Results given in Table III show the effects of chlorpromazine on glycine metabolism in a saline–phosphate–glucose medium when the respiration of cortex slices was increased by the addition of either KCl (100 m*M*) or 2,4-dinitrophenol (DNP) (0.025 m*M*). The control values (in the absence of chlorpromazine) showed that although KCl and DNP markedly increased the rate of oxygen uptake, they had little effect on glycine breakdown to CO_2 and they inhibited markedly the rate of glycine incorporation. Chlorpromazine, at 0.3 m*M*, and 0.6 m*M*, progressively inhibited the rates of glycine metabolism as well as the rates of oxygen uptake in presence of KCl or DNP. Comparison of the inhibitory effects of chlorpromazine on the metabolism

TABLE III

EFFECTS OF CHLORPROMAZINE (CP) ON GLYCINE-1-C^{14} METABOLISM AND OXYGEN UPTAKE BY RAT BRAIN CORTEX SLICES IN PRESENCE OF EITHER KCl (100 m*M*) OR DINITROPHENOL (DNP) (0.025 m*M*)
(Saline–phosphate–glucose medium. Time = 90 min.)

Stimulant added	CP, m*M*	Glycine incorporation per mg. protein		$C^{14}O_2$ formed per mg. dry tissue		Oxygen uptake per mg. dry tissue	
		c.p.m.	% inhibition	c.p.m.	% inhibition	μl. O_2	% inhibition
KCl	0	8.8	—	625	—	28.6	—
KCl	0.3	6.8	23	617	2	21.8	24
KCl	0.6	4.0	55	308	51	13.2	54
DNP	0	18.0	—	733	—	29.0	—
DNP	0.3	5.7	69	383	47	18.0	38
DNP	0.6	3.5	81	141	80	8.3	71

of potassium-stimulated and DNP-stimulated rat brain cortex tissue indicated that the latter process was apparently more sensitive to the action of the drug.*

Effects of Amytal on Glycine Metabolism in the Presence of Glucose and Rat Brain Cortex Slices

Some experimental results obtained with amytal are given in Table IV for comparison with those obtained with chlorpromazine. It will be observed that the presence of amytal brought about inhibitions of the rates of glycine incorporation and of glycine breakdown to CO_2. The percentage inhibition increased from 4 to 85% with the increase of the concentration of the narcotic from 0.1 m*M* to 1.0 m*M*. The magnitude of this narcotic inhibition was similar to that produced by 0.2–0.6 m*M* chlorpromazine during a similar experimental period of time.

TABLE IV

EFFECTS OF AMYTAL ON GLYCINE-1-C^{14} METABOLISM AND OXYGEN UPTAKE BY RAT BRAIN CORTEX SLICES (IN A SALINE–PHOSPHATE–GLUCOSE MEDIUM) (Time = 90 min.)

	Glycine incorporation per mg. protein		$C^{14}O_2$ formed per mg. dry tissue		Oxygen uptake per mg. dry tissue	
Amytal, m*M*	c.p.m.	% inhibition	c.p.m.	% inhibition	µl. O_2	% inhibition
0	43.0	—	808	—	17.7	—
0.1	41.3	4	767	5	17.2	3
0.5	19.5	55	441	46	11.6	34
1.0	6.3	85	120	85	4.8	73

Discussion

It is evident that chlorpromazine is effective in diminishing the incorporation of glycine-1-C^{14} into the proteins of rat brain cortex slices at the low concentrations that have little or no effect on the unstimulated brain respiration. The presence of 0.2 m*M* chlorpromazine brings about 20% inhibition of glycine incorporation in rat brain cortex slices respiring in a glucose–phosphate–saline medium. Higher concentrations are correspondingly more effective.

Inhibition of glycine incorporation into the proteins of brain cortex is a sensitive index of chlorpromazine activity *in vitro*. It is known that the incorporation of glycine into tissue proteins is an energy-requiring process and it is assumed that adenosine triphosphate is involved, as "uncoupling" agents such as dinitrophenol inhibit such incorporation. The inhibitive effect of chlorpromazine would indicate that "uncoupling" may occur at concentrations of the drug that have but little effect on the oxygen uptake

*Results that we hope to publish shortly show that an antagonism between potassium ions and glycine, so far as glycine-1-C^{14} incorporation into brain proteins is concerned, seems to occur. This would account (at any rate partly) for the lowered rate of incorporation of glycine-1-C^{14} into brain proteins in presence of increased potassium ion concentrations.

of unstimulated brain cortex. Abood (1) has pointed out that chlorpromazine at low concentrations "uncouples" oxidative phosphorylation of brain mitochondria and Century and Horwitt (4) have obtained a similar result with brain homogenates, pointing out that mitochondria may be more sensitive to the drug than homogenates or slices. Bersohn *et al.* (3) have also shown "uncoupling" effects of low concentrations (0.25 m*M*) of chlorpromazine with rat brain mitochondria. On the other hand, Berger *et al.* (2) could show no uncoupling effect of chlorpromazine (0.2 m*M*) on brain mitochondria (in contrast to liver mitochondria) under their experimental conditions. It is of interest to note that Dianzani and Scuro (5) have found that a variety of dyestuffs brings about inhibitions of oxidative phosphorylation with mitochondria preparations.

A preliminary note by Magee *et al.* (7) has shown that the addition of chlorpromazine (0.1 m*M*) to slices of guinea pig brain respiring in an appropriate glucose–Ringer solution, containing radioactive inorganic phosphate, causes an increase in the specific activity of the lipid phosphorus, the increase occurring mostly with the inositol-P compound. There was a decrease in the labelling of the phosphatidylethanolamine and phosphatidylcholine. At higher concentrations of chlorpromazine there was a considerable decrease in the labelling of lipid P and a fall in the concentrations of creatine phosphate and adenosine triphosphate.

Our results also show that chlorpromazine at low concentrations (0.2 m*M*) affects phenomena in the brain cell involving energy of respiration, possibly by an "uncoupling" process, and that this effect may be demonstrated with brain cortex slices. It is possible that this technique, of studying the action of chlorpromazine on glycine incorporation into the proteins of intact brain tissue, may be used with profit in the study of the effects of depressants *in vitro*. It is, clearly, of importance to investigate the action of the drug on brain tissue as near the physiological state as possible and the present technique offers, therefore, some advantages over the study of brain homogenates and mitochondria.

Acknowledgments

Grateful acknowledgment is made to the Foundations' Fund for Research in Psychiatry (New Haven) and to the National Research Council of Canada for grants which made this work possible.

References

1. Abood, L. G. Proc. Soc. Exptl. Biol. Med. **88**, 688 (1955).
2. Berger, M., Strecker, H. J., and Waelsch, H. Nature, **177**, 1234 (1956); Ann. N.Y. Acad. Sci. **66**, 806 (1957).
3. Bersohn, J., Namajuska, I., and Cochrane, L. S. G. Arch. Biochem. Biophys. **62**, 274 (1956).
4. Century, B. and Horwitt, M. K. Proc. Soc. Exptl. Biol. Med. **91**, 493 (1956).
5. Dianzani, M. U. and Scuro, S. Biochem. J. **62**, 205 (1956).
6. Lindan, O., Quastel, J. H., and Sved, S. Can. J. Biochem. and Physiol. **35**, 0000 (1957).
7. Magee, W. L., Berry, J. F., and Rossiter, R. J. Biochim. et Biophys. Acta, **21**, 408 (1956).

A "CAT AND MOUSE TEST" FOR STUDYING CHANGES IN CONFLICT BEHAVIOR[1]

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Abstract

A method is described for producing "conflict behavior patterns" in the cat, which is useful in studying the effect of drugs on conditioned reflexes. It yields data from which an ED_{50} may be calculated. Comparison of four ataractic agents by this procedure demonstrates the same relative order of activity as obtained by more complicated methods.

Introduction

Many tests have been described for the study on the effect of therapeutic agents on conditioned responses in various species of animals (Holten and Sonne (3), Jacobsen and Skaarup (4), Cook and Weidley (2), Smith *et al.* (6)).

Such methods employ the technique of conditioning animals against a noxious stimulus, thereby producing what has been described as a "conflict behavior pattern". The main difficulty with these tests, rendering them inconvenient as a screening procedure, is the relatively long period of training or conditioning required.

We have employed a simple procedure for studying the action of various agents on such conflict behavior in the cat. The method has the advantage of requiring a short training period and yet yielding quantitative data comparable to that obtained by more involved procedures.

Methods

Adult cats are chosen because of their natural hostility to mice. Such animals are then conditioned against mice by an electric current applied whenever the mouse is attacked. An automobile spark coil and a 6-volt battery supplies the current. The test is conducted on a concrete floor with one lead earthed. The second lead is attached to the tail of the mouse by means of a clip. When the cat attempts to pick up the mouse the current is applied by the operator.

Some animals respond to this treatment in such a way that they will not touch a mouse under any circumstances after only one such experience. On the average, three exposures are required and the avoidance behavior will persist for several weeks without reinforcement. In a series of 10 cats it was observed that none required further conditioning for 3 weeks.

When certain ataractic agents are administered to these cats, despite repeated electric shocks, they will continue to attack the mouse. The cat feels the shock for the mouse is dropped but is picked up again and again.

[1] Manuscript received July 10, 1957.
Contribution from the Research Laboratories, Frank W. Horner Ltd., Montreal, Quebec.

The standard method employed is to expose each cat three times on each occasion, and the number of times the animal attacks the mouse is recorded. These data expressed as percentages are recorded at various dose levels. From these dose–response curves an ED_{50} can be established (Litchfield and Wilcoxon (5)).

Results and Discussion

Table I summarizes the results obtained by this method with four ataractic agents. Benactyzine, chlorpromazine, and Histol (β-dimethylaminoethyl *p*-chloro-α-methyl benzhydryl ether hydrochloride) were observed to be the most potent and of a similar order of activity. Meprobamate was the least active in altering this conflict behavior pattern. The relative inactivity of meprobamate in inhibiting conditioning has been reported by Berger (1).

These results demonstrate the same relative order of activity of these ataractics as is obtained by more involved methods and illustrates the usefulness of this "cat and mouse test" as a screening procedure.

TABLE I

THE EFFECT OF ATARACTIC AGENTS ON THE CONFLICT BEHAVIOR PATTERN IN THE CAT. AGENTS ADMINISTERED 15 MINUTES BEFORE TESTING BY THE INTRAPERITONEAL ROUTE

Compound	No. tests	Dose, mg./kg.	Response, %	ED_{50} mg./kg. (95% fiducial limits)
Benactyzine	6	2.5	33	3.3
	12	5.0	75	(2.3 – 4.7)
	6	10.0	100	
Chlorpromazine	6	2.0	0	
	9	5.0	55	4.7
	9	10.0	90	(3.2 – 6.8)
Histol	3	2.5	0	
	6	5.0	50	5.0
	6	10.0	100	(4.2 – 5.55)
Meprobamate	3	5.0	0	
	6	10.0	17	19.0
	9	20.0	55	(12.6 – 28.5)

Acknowledgments

We wish to thank Poulenc, Ltd., Montreal, for the chlorpromazine and Wallace Laboratories, New Brunswick, N.J., for the meprobamate used in these experiments.

References

1. BERGER, F. M. Ann. N.Y. Acad. Sci. **67**, 685 (1951).
2. COOK, L. and WEIDLEY, E. Ann. N.Y. Acad. Sci. **66**, 740 (1957).
3. HOLTEN, C. H. and SONNE, E. Acta Pharmacol. Toxicol. **11**, 148 (1955).
4. JACOBSEN, E. and SKAARUP, Y. Acta Pharmacol. Toxicol. **11**, 125 (1955).
5. LITCHFIELD, J. T. and WILCOXON, F. J. Pharmacol. Exptl. Therap. **96**, 99 (1949).
6. SMITH, R. P. *et al.* J. Pharmacol. Exptl. Therap. **119**, 317 (1957).

THE RELATION BETWEEN HEART RATE AND VAGAL STIMULATION FREQUENCY IN THE RAT AT DIFFERENT BODY TEMPERATURES[1]

G. W. MAINWOOD

Abstract

The peripheral end of the right vagus was stimulated in acutely vagotomized rats under anaesthesia at different body temperatures. A fairly abrupt increase in the *R–R* time on the electrocardiogram was observed when a critical stimulation frequency was reached. At a body temperature of 35° C. the critical frequency is 10 per second or more. On lowering of the body temperature to 28° C. the critical frequency is greatly reduced so that considerable slowing may be elicited at frequencies as low as one to five per second. Possible theoretical models to account for the critical frequency and its variation with temperature are considered. The temperature effect is too great to be accounted for either on the basis of the increased number of impulses which reach the heart per cardiac cycle, or the reduced rate of cholinesterase activity. The theory most consistent with the results is that each vagal impulse liberates or inactivates a substance, the concentration of which varies directly with heart rate. The interimpulse interval at the critical frequency would then represent the regeneration time of this substance. The temperature coefficient of the regeneration process appears to be about 3.3 per 10° C.

Introduction

The liberation of acetylcholine at vagal nerve endings in the pacemaker region has long been associated with the bradycardia ensuing upon vagal stimulation (12). The effect of acetylcholine and vagal stimulation on heart rate may be varied and even reversed under the appropriate conditions (10, 4).

One variable condition which has received comparatively little attention in this connection is temperature. The effect of temperature on cardiac vagal inhibition is of interest from two points of view. First, any change in the response of the pacemaker receptors must have an effect on the whole reflex 'feed back' system, which may be of considerable significance in general hypothermia and in recovery from extreme hypothermia, which is essentially a problem of cardiac activation (1). It has in fact been suggested, though with little supporting evidence, that the fibrillation and bradycardia of hypothermia are not due to temperature per se but to the secondary effect of acetylcholine (13). Secondly the study of the effect of temperature on the response of the heart may provide information concerning the mechanism of vagal action on the heart.

In order to interpret changes in heart rate as a transmitter–receptor interaction, it is necessary to consider the theoretical relation between stimulation frequency and its effects.

In the experiments to be reported the effect of vagal stimulation on heart rate was examined at different body temperatures in anaesthetized rats. The results obtained will be considered in the light of possible theoretical models.

[1]Manuscript received May 3, 1957.
Contribution from the Department of Physiology, University of Ottawa, Ottawa, Ontario.

Materials and Methods

Young male albino rats of 50 to 100 g. weight were used in all experiments. Electrocardiograph (e.c.g.) recordings were made with a Sanborn visocardiette. A Grass model S4 C stimulator was used together with an isolation unit for vagal stimulation. Small hooked silver electrodes were used for stimulating the vagus.

The animals were given an intraperitoneal injection of Dial (Ciba) (0.65 ml./kg.) about 15 to 20 minutes before the operative procedure. Both vagi were exposed and freed from the surrounding tissues by blunt dissection. After ligation they were both cut and the peripheral end of the right vagus was placed on the hooked electrodes. The nerve and electrodes were insulated from the surrounding tissues by thin waxed paper and were washed with Tyrode solution between each period of stimulation. Initial experiments indicated that a maximal effect was practically always obtained when a stimulus of 1 volt was applied for a duration of 1 millisecond. In most experiments, to ensure maximal stimulation a pulse of 5 volts was used. The stimulation pulses were monitored on an oscilloscope connected to the electrodes.

The Lead II e.c.g. was taken through intramuscular hypodermic needles. During periods of stimulation a dipole of about 0.5 to 1.5 volts was applied to the skin of the animal through one pole of a two pole switch which operated the stimulator. This caused a deflection of the baseline of the e.c.g., so marking the beginning and end of stimulation. A standardized procedure was developed from which most of the results described here were obtained. In this procedure an e.c.g. was taken and the body temperature brought to a standard low level of 28° C. before the vagi were severed. The e.c.g. was again taken at this temperature before and after the nerves were cut. Cooling was effected by blowing a stream of air over the wettened body surface of the animal. Stimulation of the peripheral end of the right vagus was then started at a frequency of one per second. An e.c.g. was taken immediately before and during the beginning of stimulation. When the heart appeared to be beating steadily during the stimulation, generally after 15 to 20 seconds, the e.c.g. was again switched on for a few seconds and the end of the stimulation period was recorded. After a 1 to 2 minute rest and washing of the nerve with Tyrode solution, the heart was again stimulated at a frequency of two per second. Subsequent series of stimulations at 4, 8, 12, and 16 per second were made and occasionally at higher frequencies. The rat was then warmed by radiant heat and the whole procedure was repeated at a body temperature of 35° C.

Results

The normal resting temperature of the anaesthetized rats was about 35° C.; at this temperature stimulation had comparatively little effect below frequencies of 8 to 10 per second. Slowing generally appears fairly suddenly at a frequency above this level and increases up to stimulation rates of 40 to

60 per second. As the body temperature was lowered the slowing effect could be observed at progressively lower frequencies and the slope of the frequency effect curve was considerably increased beyond the inflexion point. The frequency at which the inflexion occurs will hereafter be called the critical frequency. Fairly typical results from two experiments may be seen in Fig. 1.

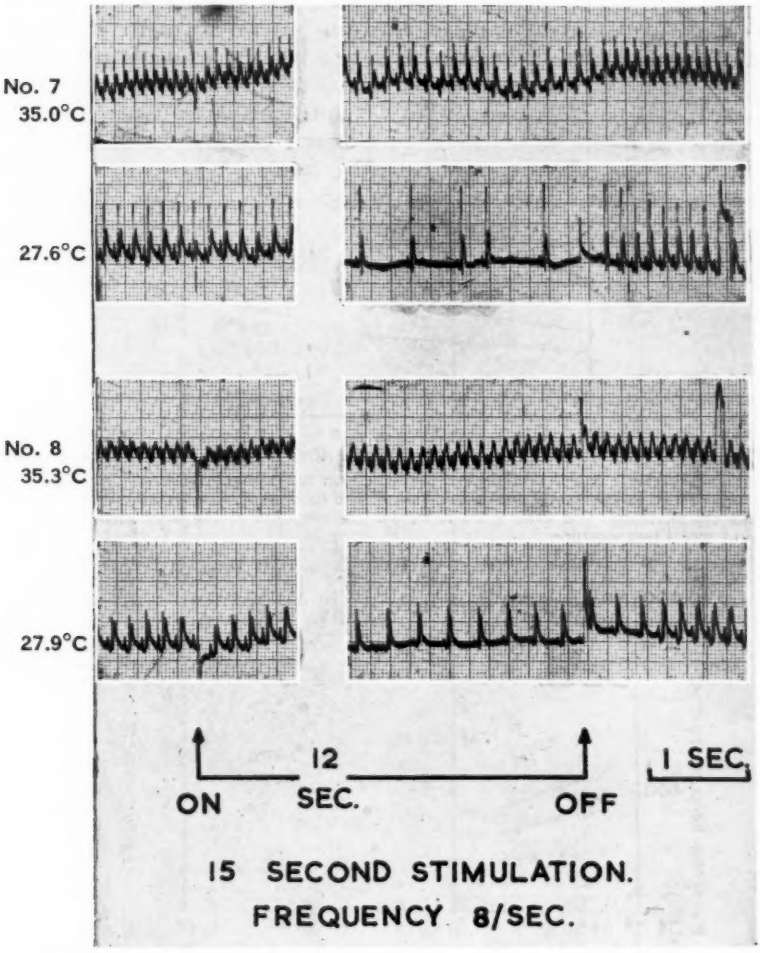


FIG. 1. The response of the e.c.g. to stimulation of the right vagus in the rat and the modification of this response during hypothermia. Both rats (Nos. 7 and 8) were anaesthetized with Dial and bilaterally vagotomized before the recordings were made. The rectal temperature at the time of the recording is indicated on the left hand side. A 1 millivolt calibration signal is included at the right hand end of the second and third lines. The total duration of the stimulation period marked by the arrows was 15 seconds. The gap in the e.c.g. recording occupies a 12 second period during which the final state was established.

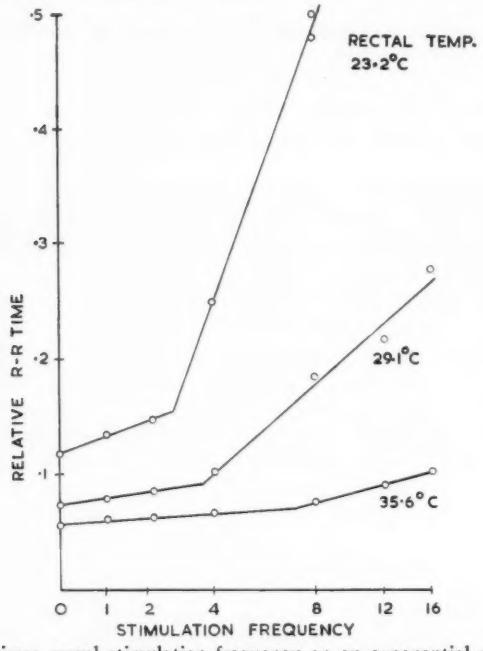


FIG. 2. Abscissa: vagal stimulation frequency on an exponential scale. Ordinate: the reciprocal of heart rate or mean duration of the cardiac cycle after about 15 to 20 seconds stimulation. The three lines represent the response of the same rat at three different rectal temperatures.

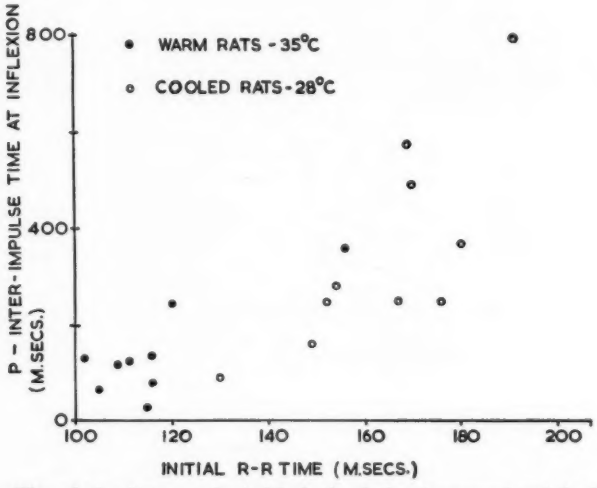


FIG. 3. The relation between the 'critical stimulation frequency' and the initial, pre-stimulation *R–R* time. Abscissa: mean *R–R* time immediately before stimulation. Ordinate: the reciprocal of stimulation frequency or mean interimpulse interval at the critical frequency.

In these rats, at normal body temperature, slowing is barely perceptible at a frequency of 12 per second; in the same rats at 27° to 28° C. however, the response is considerable with a slowing of 50% or more.

In another experiment (Fig. 2) stimulation over a range of 1 to 16 per second was performed first at a rectal temperature of 29° C., then at 23° C., and finally at 35.6° C. This figure shows the inflexion point, or critical frequency of the frequency effect curves and its variation with temperature. Such curves were observed regularly and the same temperature effect occurred whether the animal was first warm and then cold or vice versa.

The effect of vagal stimulation did show considerable variation from rat to rat even at a fixed body temperature. The critical frequency appeared to vary in such a way that it was correlated with the initial prestimulation heart rate. On applying a small number rank correlation test (14) to the data

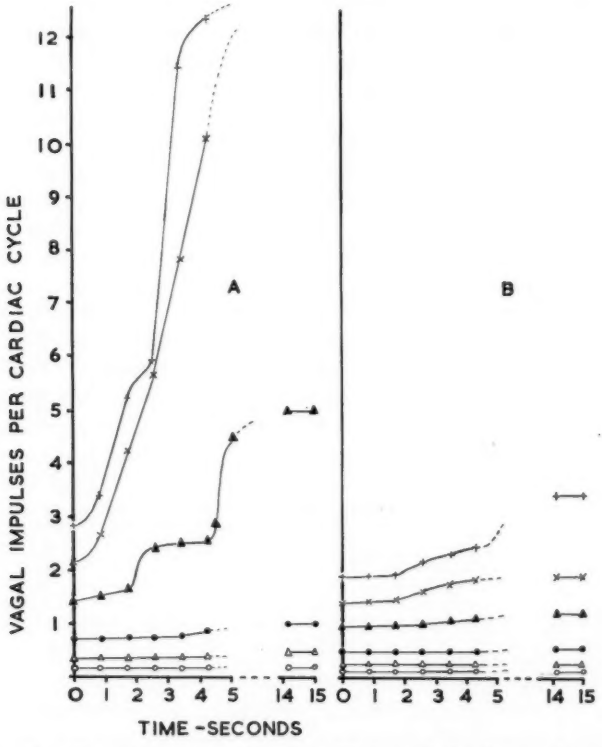


FIG. 4. The effect of vagal stimulation at different relative frequencies on the time course of the e.c.g. response. Abscissa: time in seconds from the beginning of stimulation. Ordinate: the relative number of vagal impulses per cardiac cycle. A: the response of a rat at a rectal temperature of 28° C.; B: the response of the same rat at a rectal temperature of 35° C. Stimulation frequencies: open circles, 1 per second, open triangles, 2 per second; black circles, 4 per second; black triangles, 8 per second; and oblique crosses, 12 per second; upright crosses, 16 per second.

in Fig. 3, P values of .01 for all rats and .05 for cold rats alone were given, so that it seems reasonable to conclude that there is a relation between heart rate and the critical frequency beyond their dependence on temperature.

The possibility that the effect may be explained on a relative basis was considered, i.e. that the degree of slowing depended upon the number of vagal impulses reaching the heart per cardiac cycle. If so, then the degree of slowing at a fixed frequency would be dependent on the initial length of the cardiac cycle which increases as the body temperature falls. The effect of stimulation at different initial relative frequencies is seen in Fig. 4.

It is evident that with this correction there is still a very big difference between the response of the warm and the cooled rats.

If we suppose that the effect of each impulse is eliminated during a particular phase of the cardiac cycle then comparatively little summation would occur until a frequency was reached at which two impulses occurred within the same cycle. Under certain conditions we may then expect a 'run away' type of inhibition with the number of vagal impulses and therefore the effect increasing as the cardiac cycle lengthens.

This type of inhibition does sometimes appear to occur as in the cold rat in Fig. 4. At a stimulation frequency of four per second a steady state of just one impulse per cycle was finally reached. This appeared to be the critical frequency from interpolation of the frequency effect curve. At frequencies much above this, the slowing was such that it soon reached a level of two impulses per second, which further accelerated the slowing; this occurs again at three impulses per second.

Data derived from the mean steady state heart rate probably give more reliable information than the time response curve because of the variability of the individual cycle lengths and the relatively rapid onset of the inhibition.

Mean values of some of these data derived from 10 rats following the standardized procedure given above are presented in Table I.

TABLE I

MEAN R–R TIME IN ANAESTHETIZED, VAGOTOMIZED RATS AND THE ESTIMATED RECOVERY TIME ($1/F_c$) AT TWO DIFFERENT TEMPERATURES

	Cooled rats		Warm rats		Ratio	Significance level
	Mean	S.E.M.	Mean	S.E.M.		
Rectal temp.	28.02	± 0.14	34.89	± 0.06		
Mean R–R time	163.8	± 5.07	115.8	± 4.81	1.42	<0.01
$1/F_c = A/K_e$	355.8	± 67.3	136.1	± 34.9	2.62	<0.01

Discussion

The curves relating vagal stimulation frequency to heart rate show an inflexion at a critical stimulation frequency, which becomes sharper as the temperature is lowered.

The shape of these curves may most easily be interpreted by assuming that each vagal impulse has an effect which decays with time in a linear or nearly linear fashion. An abrupt increase in response would then be effected when the interval between impulses just exceeds the time required for the elimination of its effect.

Although in some cases in cooled animals a single vagal impulse has some effect, it is not possible to estimate accurately the time course of the individual response. Heart slowing by single vagal impulses has, however, been investigated in some species which show a more pronounced response.

An analysis of the time course of the inotropic effect of a single vagal shock on the turtle atrium was presented by Gilson (9). Single shock response curves have also been investigated in the cat heart (3). The effect showed a sharp rise after the arrival of the impulse followed by a gradual decay which may in the first approximation be considered linear.

In these experiments the heart frequency under steady state conditions may be related to the individual impulse response by a simple kinetic model, from which the relative heart rates at different impulse frequencies may be calculated (Appendix I).

The marked temperature effect observed here is of interest in that it provides a means of further analyzing the rate-limiting stage in the sequence of events leading to a slowing of the pacemaker. Of the three distinct stages involved in this sequence, namely, impulse conduction, humoral transmission, and the pacemaker response, the second has been generally considered as determining the time course of the effect (9, 3). Curves with a similar inflexion to those found here have been observed in sympathetic vasomotor responses; these have also been attributed to a rate-limiting process in the humoral transmission phase (7, 8). There is, however, as yet no conclusive evidence as to which stage predominates in determining the time course of the effect. The evidence for each of the three possibilities will be considered here in turn.

Firstly, the conduction stage could determine the time course of the response under one of the following circumstances. (1) The asynchronous arrival of action potentials at the nerve endings over a period of 100 milliseconds or more. Such a circumstance could only arise in the presence of ganglion delay mechanisms since the conduction path is too short to cause any considerable spreading due to different conduction velocities. (2) A continued liberation of acetylcholine at the nerve endings for a period over a hundred times greater than the duration of the action potential.

Although it has been claimed that the delayed liberation of acetylcholine may last for a period of over 100 milliseconds in the frog nerve ending (6), the evidence is indirect and does not appear to justify the conclusion. It seems much more likely that the duration of the period of acetylcholine liberation lasts for only a fraction of a millisecond.

The correlation between critical frequency and the initial prestimulation *R–R* time (Fig. 3) is the only evidence in this work which can be presented against the possibility that the rate limiting factor is in the cardiac nerve

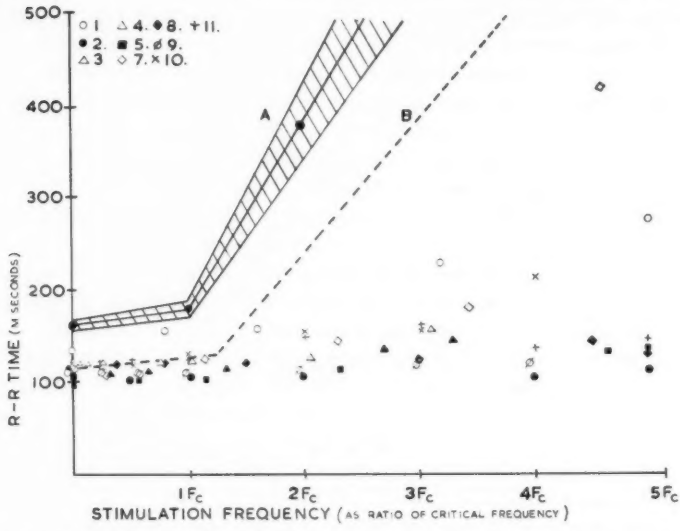


FIG. 5. The mean critical frequency for a group of 10 rats at a body temperature of 28° C. is taken as F_c. Curve A represents the response of this group of rats derived from measurements of the mean R–R time during stimulation at the critical frequency, at twice the critical frequency, and before stimulation. A measure of the scatter of these results is given by the standard error of the mean indicated by the area shaded with vertical lines. Line B is calculated from this on the assumption that the decreased relative response at 35° C. is entirely due to an increased cholinesterase activity having a temperature coefficient of 0.3/10° C. in this range. The actual response of each of the same 10 rats at this temperature at multiples of the critical stimulation frequency is shown by the symbols identified as rat numbers in the top left hand corner.

network. The application of the Olmstead and Tuckey association test (14) shows that this correlation is significant even if the temperature is fixed at 28° C. Thus if the critical frequency is dependent on delayed acetylcholine release in the nerve, one would have to suppose that the function of the effector nerve is controlled by its effector organ or that the heart beat is neurogenic.

If the rate-limiting step is in fact to be found in the humoral transmission stage it must be determined by the rate of breakdown of the transmitter substance, presumably under the action of cholinesterase. This possibility may readily be tested by a comparison of the temperature characteristics of the observed system with those which would be expected if the rate of decay of each effect were determined by cholinesterase activity. It may be seen in Fig. 5 that in every one of 10 rats the vagal effect at 35° C. was far less than that predicted from the extent of the effect at 28° C. if the temperature coefficient of cholinesterase alone were the factor responsible for the decreased response. An alternative approach is to calculate the energy of activation which the transmitter breakdown reaction must have if it is to satisfy the observed conditions. It may be seen from Table II that the required activation energy is about four to five times greater than that involved in the breakdown of acetylcholine by cholinesterase.

Before excluding cholinesterase as the rate-limiting factor it is necessary to consider the assumptions involved in predicting the relative slowing in the model system. First it was assumed that the amount of acetylcholine liberated at each impulse does not vary with temperature. If this does decrease to any considerable extent with increasing body temperature then these conclusions would be invalid.

Kostial and Vouk have shown that in the superior cervical ganglion there is no significant effect of temperature on the acetylcholine release from nerve endings in the range of 20 to 40° C. (11). Using a frequency of 10 per second as opposed to two per second by the above authors, Brown (2) did show a temperature effect but this was in the opposite direction to that necessary to cause a change in the critical frequency, i.e. the acetylcholine output increased with rising temperature. It seems then unlikely that a change in the amount of acetylcholine liberated could contribute to the observed effect.

TABLE II

THE APPARENT ACTIVATION ENERGY OF THE RECOVERY PROCESS COMPARED WITH OTHER ACTIVATION ENERGIES

The observed activation energy is calculated from the Arrhenius equation on the assumption that the change in critical frequency is due entirely to a change in K_r in the temperature range of 28–35° C.
Other activation energies are taken from the work of Wilson and Cabib (17).

	Approx. activation energies, cal./mole
Observed	25,000
Cholinesterase *in vitro*	5,500
Base-catalyzed ACh breakdown	14,000
Cholinesterase with deacetylating system	15,000–20,000

A second assumption is that the intracardiac cholinesterase has similar properties to cholinesterase preparations examined *in vitro*. In this connection the recent work of Wilson and Cabib (17) provides some interesting possibilities. They consider the cholinesterase reaction to be a two-stage process in which the deacetylation of the enzyme following the splitting off of choline is the rate-limiting reaction at higher temperatures. The ester hydrolysis has a much greater activation energy than the deacetylation but this is masked by the slower secondary reaction. Conceivably the apparent temperature coefficient of cholinesterase could be increased if it were combined with an appropriate deacetylating system *in vivo*. Even under these conditions the temperature coefficient of the vagal effect appears to be too great to be accounted for by cholinesterase activity alone (Table II).

We are then left with the third possibility that the response of the pacemaker itself to the transmitter substance must be affected by temperature. If the transmitter substance causes a slowing of the pacemaker it must do so either by combining with active groups on the receptor surface (15) or by causing a

loss of some element as in the outward diffusion of ions from (5) or inactivation of some groups in the pacemaker which themselves are important in impulse generation. The recovery from the effect of an impulse would then be due to the regeneration of these active groups or ions by dissociation, synthesis, ion pumps, or some other restoring process. Whatever the specific process, a simple model presents itself which may be used to test quantitatively this general theory. If we assume that the pacemaker rate bears a constant relationship to the concentration of some hypothetical element and that one unit of transmitter substance inactivates a certain fraction of this element in the pacemaker then it may be shown that:

$$R\text{–}R \text{ time (stimulated)} = (F_x/F_c)\ R\text{–}R \text{ time (unstimulated)}.$$

In Table III this is compared with the predictions based upon the second theory (Appendix I). It may be seen that the mean values of the observed results show a fairly good quantitative agreement with the third theory outlined above. The observed correlation between initial prestimulation heart rate and critical frequency (Fig. 3) also lends support to this theory since the inherent rate of production of the pacemaker element may determine its steady state level in the normal unstimulated heart as well as the regeneration time following its loss subsequent to a vagal impulse.

The data of Gilson (9) lend some support to these observations. In fitting the appropriate curve to the negative inotropic effect of vagal stimulation in the turtle atrium between 16° and 22° C. the respective apparent velocity constants for the breakdown of the transmitter substance are found to be 0.05 and 0.08, which would give a fractional increase of 1.0 per 10° C. as opposed to 0.3 per 10° C. for cholinesterase activity at this temperature. This does tend to indicate that cholinesterase activity is not the limiting factor though the temperature coefficient which they observe is considerably lower than that found here. This is not surprising considering they were using a different species at a different temperature and measuring the inotropic and not the chronotropic effect.

In its present non-specific form this model could be interpreted in many ways. These observations may be compatible with the idea that the slowing is due to an ion loss from the pacemaker with subsequent restoration by a

TABLE III

A COMPARISON OF THEORIES 2 AND 3 (SEE TEXT) ASSUMING A RECOVERY PROCESS WITH AN ACTIVATION OF ENERGY OF ABOUT 25,000 CAL./MOLE USING THE MEAN OF THE OBSERVATIONS ON 10 RATS

	Theory 2	Theory 3	Observed
R–R time as a function of F_x	$R_0 + \frac{R_0 K_s K_x}{K_D}\left(\frac{F_x}{F_c}-1\right)$	$\frac{R_0 F_x}{F_c}$	
R–R time when $F_x = 2F_c$ at 35° C.	497.8	259.1	253.7
Relative fractional slowing at 35° and 28° C. when $F_x = 2F_c$	2.62	1.0	0.94

pumping mechanism since such mechanisms do appear to have a high activation energy. This must remain a tentative suggestion, however, without more direct evidence.

The main deviation from expectations is in the frequency-jumping effect sometimes seen during steady stimulation at rates near the heart frequency. It is possible that while the particular theory described does account for the over-all effect there is superimposed upon it a sensitivity variation throughout the cardiac cycle which would account for sudden changes when the stimulus impulse becomes synchronous with a more sensitive phase of the cardiac cycle.

Acknowledgments

I am much indebted to Dr. A. B. L. Beznák for his guidance and stimulating interest. My thanks to Dr. K. Laidler, for his useful advice. Mr. G. Jakerow and Mr. M. Colonnier took part in the work and it is a pleasure to thank them. This work was supported by a grant from the National Research Council.

References

1. ANDJUS, R. K. and SMITH, A. U. J. Physiol. **128**, 446 (1955).
2. BROWN, G. L. J. Physiol. **124**, 26P (1954).
3. BROWN, G. L. and ECCLES, J. C. J. Physiol. **82**, 211 (1934).
4. BURN, J. H. and VANE, J. R. J. Physiol. **108**, 104 (1949).
5. DEL CASTILLO, J. and KATZ, B. J. Physiol. **129**, 48P (1955).
6. DUN, F. XX Intern. Physiol. Congr. 1956. p. 259.
7. FOLKOW, B. Acta Physiol. Scand. **25**, 49 (1952).
8. FOLKOW, B. Physiol. Revs. **35**, 629 (1955).
9. GILSON, A. S. Am. J. Physiol. **100**, 459 (1932).
10. HOFFMAN, F., HOFFMAN, E. J., MIDDLETON, S., and TALESNIK, J. Am. J. Physiol. **144**, 189 (1945).
11. KOSTIAL, K. and VOUK, V. B. J. Physiol. **132**, 239 (1956).
12. LOEWI, O. Pflüger's Arch. ges. Physiol. **189**, 239 (1921).
13. NOEL, W. Arch. Psychiat. Nervenkrankh. **118**, 29 (1945).
14. OLMSTEAD, P. S. and TUCKEY, J. W. Ann. Mathematical Statistics, **18**, 495 (1947).
15. ROSENBLUETH, A. The transmission of nerve impulses at neuroeffector junctions and peripheral synapses. 1950.
16. WILSON, I. B. and CABIB, E. J. Am. Chem. Soc. **78**, 202 (1956).

Appendix I

Assuming a linear rate of decay then summation will not occur until the critical frequency (F_c) is reached when:

$$(1/F_c) = (A/K_e),$$

where A is the extent of each effect and K_e the decay constant. In this specific case A represents the amount of acetylcholine liberated per impulse and K_e the velocity of its zero order breakdown by cholinesterase.

The rate of accumulation of residual (R) at stimulation frequency F_z will be:

$$R = K_e[(F_z/F_c) - 1].$$

Let the steady state level of the transmitter substance be C at frequency F_z, then:

$$R = CK_D,$$

where K_D is a removal constant and may represent diffusion.

If K_s is the coefficient linking concentration to effect such that:

$$\frac{\Delta\ R\text{–}R\ \text{time}}{\text{Initial}\ R\text{–}R\ \text{time}} = K_sC,$$

then

$$\frac{\Delta\ R\text{–}R\ \text{time}}{\text{Initial}\ R\text{–}R\ \text{time}} = (K_sK_c/K_D)\ [(F_x/F_c) - 1].$$

Appendix II

A fraction AK_1 of the total active pacemaker element (M_t) is effectively removed by an impulse of intensity A. Then at the critical frequency (F_c), where K_2 is a zero order regeneration constant:

$$K_1AM_t/K_2 = 1/F_c.$$

If M_n is the steady state level of the element during stimulation at a frequency F_x then:

$$K_1M_nA = K_2/F_x$$

and

$$M_n = M_t(F_c/F_x).$$

If the duration of the R–R time is inversely proportional to the amount of M present then:

$$R\text{–}R\ \text{time}_{\text{(stimulated)}} = R\text{–}R\ \text{time}_{\text{(unstimulated)}}\ (F_x/F_c).$$

THE PREPARATION OF LECITHIN FROM L-α-GLYCERYLPHOSPHORYLCHOLINE[1]

N. H. TATTRIE AND C. S. MCARTHUR

Abstract

L-α-Lecithin (dipalmitoyl) has been prepared from L-α-glycerylphosphorylcholine by acylation with palmitoyl chloride in anhydrous chloroform in a yield of about 31%. This reaction also gives rise to two by-products having hemolytic activity. One of these, although not yet completely characterized, is chromatographically comparable with lysolecithin formed from lecithin by the action of snake venom lecithinase. The second by-product possessing hemolytic activity but chromatographically different from common lysolecithin was shown to be a monoacyl derivative of L-α-glycerylphosphorylcholine.

Introduction

Pure crystalline L-α-glycerylphosphorylcholine may be prepared readily in large amounts (7) and if it could be acylated a number of phospholipids and lecithin-like compounds of biological importance would be made available. A search of the literature reveals that complete acylation of this phosphoric diester does not occur under the conditions usually employed in this reaction. In 1924, Levene and Rolf (6) reported the synthesis of lecithins by acylation of lysolecithin with organic acid anhydrides in a mixture of chloroform and pyridine containing sodium acetate. However, the methods for the purification and identification were not sufficiently refined at that time to permit unequivocal identification of the products. Baer and Kates (3) after several attempts to prepare lecithin from their synthetic L-α-glycerylphosphorylcholine by acylation with organic acid chlorides in the presence of pyridine abandoned this route of synthesis since the products appeared to be mainly monoacyl derivatives, i.e., lysolecithins. A further attempt by Hanahan (4) to convert lysolecithins to lecithins by acylation with various organic acid chlorides and anhydrides was unsuccessful. In view of these reports it appeared that while one alcoholic group could be readily esterified the second was subject to hindrance. Since the barium salt of L-α-glycerylphosphorylethylenechlorohydrin (2) and the cadmium chloride addition compound of L-α-glycerylphosphorylcholine (1) may be completely acylated the hindrance seems to be related to the electrical state of the phosphorylcholine moiety of L-α-glycerylphosphorylcholine. This suggested that L-α-glycerylphosphorylcholine might undergo complete acylation by organic acid chlorides if the reaction were carried out in amine-free dry solvents which would allow the hydrogen chloride to combine with the phosphorylcholine group.

[1] Manuscript received July 15, 1957.
Contribution from the Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan.

Experimental

Dry crystalline L-α-glycerylphosphorylcholine (8.81 g.) prepared according to the method of Tattrie and McArthur (7) and 29 g. (*ca.* 3 molar equivalents) of palmitoyl chloride in 50 ml. of dry alcohol-free chloroform were placed in a strong glass-stoppered bottle containing glass beads. The reaction mixture, kept at a temperature of 37° in an incubator, was continuously shaken for a period of 72 hours and then transferred with chloroform to a separatory funnel. The free hydrochloric acid formed during the reaction and the excess palmitoyl chloride were removed by extraction of the chloroform solution with aqueous sodium bicarbonate solution. To facilitate separation of the chloroform and aqueous layers, small amounts of methanol were added. The aqueous methanol layer was re-extracted several times with chloroform and the combined chloroform extract, after it was dried over anhydrous sodium sulphate, was evaporated to dryness in a rotary vacuum evaporator at a bath temperature of 40°. The waxy residue was redissolved in a small amount of anhydrous chloroform and the solution was poured into anhydrous acetone to yield a white flocculent precipitate. The precipitate was collected on a glass Buchner funnel, washed with cold dry acetone, and finally washed with dry ether. After the crude product was dried *in vacuo* over phosphorus pentoxide and paraffin wax shavings, it weighed 19.64 g. This material was dissolved in dioxane at 60° and allowed to crystallize at room temperature over a period of 3 hours. The crystalline product was collected and washed with dry ethyl ether on a Buchner funnel and after it was dried it was found to weigh 12.89 g. A sample (100 μg.) was chromatographed on silicic acid impregnated paper using 30% (v/v) methanol in chloroform according to the procedure of Lea, Rhodes, and Stoll (5). Fig. 1, Sample 6, shows that this product contains five substances that react with phosphomolybdic acid to give blue spots when reduced with stannous chloride, although the major component of the mixture appeared to be lecithin since it migrated to the same position on the chromatogram as a specimen of synthetic L-α-(dipalmitoyl) lecithin (Baer) (Fig. 1, Sample 4). Two minor components appeared as spots in about the same position as that taken by lysolecithin prepared from purified egg lecithin by the action of *Agkistrodon piscivorus piscivorus* venom (Fig. 1, Sample 1) while the fourth component formed a crescent in front of the major spot. The fifth component remained at the origin. Chromatography of the mixed product after treatment with snake venom (Fig. 1, Sample 3) showed that the main component was converted to one behaving like lysolecithin formed from egg lecithin while the other components appeared to be unaltered.

Five grams of the reaction mixture was fractionated on a column containing 150 g. of silicic acid (Mallinkrodt, A. R.) using 20% (v/v) methanol in chloroform according to the procedure of Lea, Rhodes, and Stoll (5). The solvent was forced through the silicic acid bed by application of a slight pressure (5–10 cm. Hg) of nitrogen to the head of the column. Fractions of the effluent, 100 ml. in volume, were collected and each was evaporated

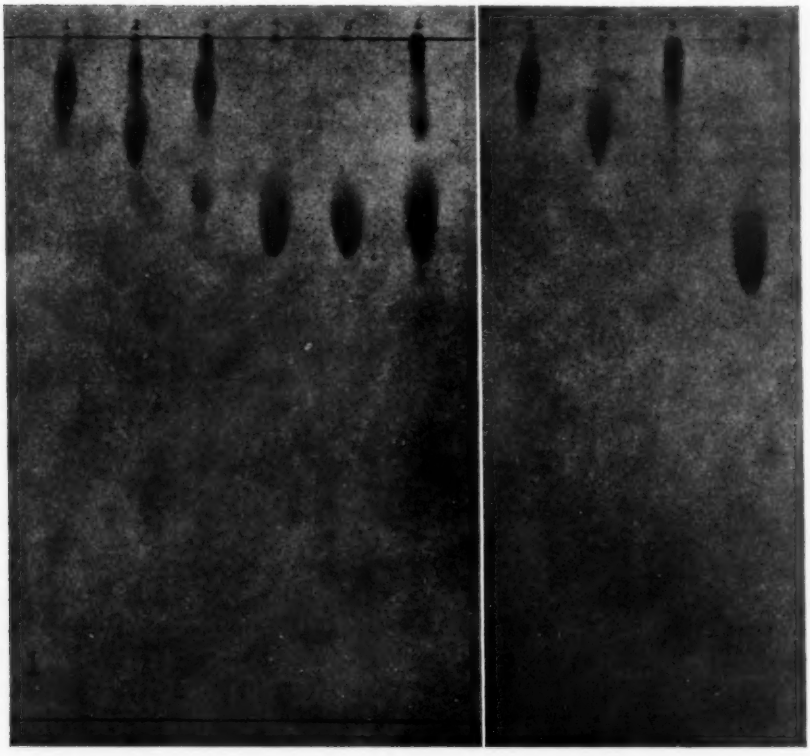


FIG. 1. Chromatogram of lipids run on silicic acid impregnated paper with methanol–chloroform mixture and stained with phosphomolybdate according to the procedure of Lea, Rhodes, and Stoll (5). (1) Lysolecithin prepared by the action of *Agkistrodon piscivorus piscivorus* venom on purified egg lecithin; (2) residual material eluted with methanol from silicic acid column; (3) original reaction mixture after treatment with snake venom; (4) authentic L-α-(dipalmitoyl) lecithin (Baer); (5) fraction of reaction mixture corresponding to L-α-(dipalmitoyl) lecithin separated by chromatography on a silicic acid column; (6) crude reaction mixture.

FIG. 2. Silicic acid paper chromatogram of venom-formed lysolecithin and two hemolytically active by-products of the acylation of L-α-glycerylphosphorylcholine with a reference sample of lecithin produced by this reaction. (1) Lysolecithin prepared from purified egg lecithin by the action of snake venom lecithinase; (2) monopalmitoyl-L-α-glycerylphosphorylcholine, which runs faster than common lysolecithin; (3) product which is chromatographically comparable with lysolecithin prepared from egg lecithin; (4) reference sample of lecithin prepared from L-α-glycerylphosphorylcholine.

to dryness in a rotary evaporator under reduced pressure. The residues, transferred to weighing bottles in a small amount of chloroform, were weighed after complete removal of the solvent. Fraction 62 (collected after 6100 ml. of the mixed solvent had passed through the column) contained only 21.2 mg. of solid matter. The material more firmly held on the column was eluted with 1200 ml. of absolute methanol. In this manner 98.3% of the original material was recovered. A portion of each fraction was submitted to chromatographic analysis on silicic acid impregnated paper. The first 14 samples (1400 ml. of effluent) containing 0.770 g. of solid matter did not contain appreciable amounts of material that could be stained on the chromatograms with the phosphomolybdate reagent. Fractions 15–39 contained 2.138 g. of solid material, samples from which, on chromatography, gave predominant spots which lay alongside that of authentic L-α-(dipalmitoyl) lecithin (Baer), with very much less dense crescents in front of the main spots. Fractions 40–62 containing 0.959 g. of solid material yielded, on chromatography, single spots which corresponded to that of authentic L-α-(dipalmitoyl) lecithin (Fig. 1, Sample 5). Fractions 15–62 contained 3.097 g. of material mainly composed of a substance which was chromatographically similar to L-α-(dipalmitoyl) lecithin, and the over-all yield from L-α-glycerylphosphorylcholine was 31%.

For purposes of identification, 1.0237 g. of this material was dissolved in a small amount of chloroform and reprecipitated from anhydrous acetone. After it was dried *in vacuo* over P_2O_5 and paraffin wax shavings the precipitate weighed 0.882 g. On analysis the following data were obtained: N, 1.86%; P, 4.25%; choline, 16.2%; palmitic acid, 68.8%. Calculated for $C_{40}H_{82}O_9NP$ (752): N, 1.86%; P, 4.12%; choline, 16.1%; palmitic acid 68.2%. Optical rotation, $[\alpha]_D^{28} + 5.5°$ in chloroform–methanol (1:1), C, 8.82. Reported (3) $[\alpha]_D^{28} + 6.6°$ in chloroform–methanol (1:1), C, 4.2.

The material eluted from the column with methanol weighed 1.047 g. and on chromatographic analysis (Fig. 1, Sample 2) was found to consist of two substances. The major component ran ahead of lysolecithin produced from egg lecithin by the action of snake venom (Fig. 1, Sample 1) while the other took up a position slightly behind the venom-formed lysolecithin. Experience in the chromatography of glycerophospholipids on silicic acid impregnated papers has shown that the distances moved by the lipid spots cannot be directly related to the apparent distance travelled by the solvent (30% v/v methanol in chloroform) front, and that the amount of lipid spotted on the paper determines to some extent the distance that the compound travels. For this reason it appears that the minor component of Sample 2, Fig. 1, corresponds to the enzyme-formed lysolecithin while the major component represents a new kind of choline-containing lipid.

A portion (0.773 g.) of the methanol-eluted material was rechromatographed on silicic acid (25 g.) in a column 3 cm. in diameter with methanol 50% (v/v) in chloroform. Fractions of the effluent having a volume of 100 ml. were collected. Small portions of the lipid in each fraction were chromatographed on silicic-acid-impregnated paper and the first fractions showing

no trace of a second component were combined. Removal of the solvent left a residue weighing 0.388 g. a sample of which on silicic acid paper chromatography gave a spot (Fig. 2, Sample 2)* which ran ahead of lysolecithin formed from egg lecithin by snake venom lecithinase (Fig. 2, Sample 1). Analysis of this product gave the following data: N : P, 1.01; choline : P, 0.98; palmitic acid : P, 0.99. Theoretical for lysolecithin: N : P, 1.00; choline : P, 1.00; palmitic acid : P, 1.00. The substance exhibited strong hemolytic activity.

The less abundant substance which was chromatographically comparable with venom-produced lysolecithin (Fig. 2, Sample 3) was difficult to purify although on a weight basis both synthetic substances and lysolecithin prepared enzymatically from egg lecithin possessed about the same hemolytic activity.

Discussion

Direct synthesis of lecithin from L-α-glycerylphosphorylcholine offers a route to the preparation of unsaturated lecithins other than that now described by Baer, Buchnea, and Newcombe (2). It might be also possible to prepare lecithins in which different fatty acids are attached at the alpha and beta positions. Hanahan (4) has presented evidence to show that snake venom lecithinase specifically removes the fatty acid from the alpha position of the lecithin regardless of the degree of unsaturation of the fatty acids. Since the results of the present investigation indicate that lysolecithins are capable of being acylated under certain conditions it may be possible to prepare lecithins with different kinds of fatty acids in the alpha and beta positions through the following sequence of reactions: (*a*) acylation of L-α-glycerylphosphorylcholine to yield lecithin containing a single type of fatty acid; (*b*) removal of the alpha-bound fatty acid moiety by means of venom lecithinase, and finally (*c*) acylation of the resulting lysolecithin with a second type of fatty acid to yield lecithin with different fatty acids in the alpha and beta positions.

One of the two main by-products of the reaction behaves in a manner similar to lysolecithin formed from lecithin by the action of snake venom lecithinase in that it is hemolytic and moves at the same rate on silicic acid impregnated paper when methanol in chloroform is used as a developing solvent. The other by-product, also possessing hemolytic activity, has the composition of lysolecithin but differs substantially from the enzyme-formed lysolecithin in its mobility when chromatographed on silicic acid containing paper. The nature of these two substances might be explained by assuming that one is β-monoacyl-L-α-glycerylphosphorylcholine while the other is α-monoacyl -L-α-glycerylphosphorylcholine. In accordance with the conclusions of Hanahan (4) regarding the structure of venom-formed lysolecithin the latter appears to be a new type of lipid.

* A sample of lecithin isolated from the reaction mixture was run as a reference substance (Fig. 2, Sample 4).

Acknowledgments

This investigation was supported by a grant from the Division of Medical Research, National Research Council of Canada, and a Summer Research Associateship (1956) to one of us (C.S.M.) from the National Research Council of Canada. The authors wish to thank Dr. Erich Baer of the Banting and Best Department of Medical Research, University of Toronto, for this kindness in supplying us with a sample of synthetic L-α-(dipalmitoyl) lecithin.

References

1. BAER, E. Can. J. Biochem. and Physiol. **34**, 304 (1956).
2. BAER, E., BUCHNEA, D., and NEWCOMBE, A. G. J. Am. Chem. Soc. **78**, 232 (1956).
3. BAER, E. and KATES, M. J. Am. Chem. Soc. **72**, 942 (1950).
4. HANAHAN, D. J. J. Biol. Chem. **207**, 879 (1954).
5. LEA, C. H., RHODES, D. N., and STOLL, R. D. Biochem. J. (London), **60**, 353 (1955).
6. LEVENE, P. A. and ROLF, I. P. J. Biol. Chem. **60**, 677 (1924).
7. TATTRIE, N. H. and MCARTHUR, C. S. Can. J. Biochem. and Physiol. **33**, 761 (1955).

THE EFFECT OF SULPHAEMOGLOBIN ON RED CELL VIABILITY[1]

L. G. Israels, A. Chutorian, G. E. Delory, and Esther Israels

Abstract

Sulphaemoglobinaemia was produced in rabbits by the injection of para-aminopropriophenone and calcium sulphide. The disappearance of this pigment from the blood was used as an index of red cell survival. Sulphaemoglobin disappeared in an exponential fashion, indicating a mean red cell life span of 36 days. The red cells were also tagged with Cr^{51}, and this method of measuring erythrocyte life span yielded values strongly suggesting that sulphaemoglobin in the red cell impairs its viability and leads to random cell destruction. Under these conditions it would seem that the disappearance rate of sulphaemoglobin is not a true measure of red cell survival.

Introduction

The determination of the life span of the erythrocyte is a useful technique both on the ward and in the laboratory. For experimental work in animals the method of differential agglutination is usually not applicable and methods involving the excretion of bilirubin are cumbersome. The use of radioactive chromium, iron, and carbon would now seem to be the methods of choice. However, as sulphaemoglobin can be easily induced in animals and the estimation of sulphaemoglobin in the peripheral blood is relatively simple, it seemed that if sulphaemoglobin remained in the red cell throughout the entire life span and did not compromise the life of the cell, this would be a valuable technique for use in experimental animals.

The interest in sulphaemoglobin as a measure of red cell life span began in 1946 when Jope (4) followed the disappearance of this pigment from the blood of seven TNT workers. The averages of the values for the seven subjects relating the amount of sulphaemoglobin as a percentage of its initial value against the time in days after exposure showed a linear disappearance of sulphaemoglobin which approached zero at approximately 116 days. This agrees well with the red cell survival studies in man using other techniques. Subsequently, other workers induced sulphaemoglobinaemia in animals, followed the disappearance of the tagged cells, and concluded that sulphaemoglobin could be used as a measure of red cell life span (5, 2, 10). None of the values obtained, however, agree with other measurements of red cell life span in the same animal species using other cell tags. In view of these apparently contradictory findings we undertook a re-examination of the problem.

[1] Manuscript received July 2, 1957.
Contribution from the Department of Biochemistry, University of Manitoba, Winnipeg, Manitoba. This work was aided by a grant from the National Research Council of Canada, and the Manitoba Cancer Institute.

Experimental

In determining sulphaemoglobin it is necessary also to determine total haem pigment in the presence of this substance. The advantage of the cyanmethaemoglobin method for the determination of total haem pigment is that most haem pigments are converted to this substance upon the addition of sodium cyanide and potassium ferricyanide. Although sulphaemoglobin undergoes a marked structural change on the addition of these two reagents it does not yield cyanmethaemoglobin. It is converted to a pigment which we shall call "cyansulphaemoglobin". Cyansulphaemoglobin has an absorption curve somewhat similar to that of cyanmethaemoglobin in the visual range but a lower peak at 540 mμ and it intersects the cyanmethaemoglobin curve at 578 mμ. When sulphaemoglobin is present the addition of potassium ferricyanide and sodium cyanide converts the sulphaemoglobin to cyansulphaemoglobin and the remaining haem pigments to cyanmethaemoglobin. The optical density of this mixture read at the isobestic point 578 mμ is a measure of total haem pigment. We used a Beckman Spectrophotometer Model DU calibrated by iron analysis. The extinction coefficient for sulphaemoglobin was determined experimentally by preparing a series of solutions containing various mixtures of haemoglobin and sulphaemoglobin. The total haem pigment was estimated as indicated above and the haemoglobin content determined by the oxygen capacity method described by Peters and Van Slyke (9). As no other haem pigment was present (specimens were examined for choleglobin) the difference between the total haem pigment and the haemoglobin content represented sulphaemoglobin. With the extinction coefficient of sulphaemoglobin known, the concentration of sulphaemoglobin in any specimen was determined from the formula:

$$C_{SHb} = \frac{(E_{Hb620} \times C_t) - D_{620}}{E_{Hb620} - E_{SHb620}},$$

where C_{SHb} = concentration of sulphaemoglobin; D_{620} = optical density of the original solution at 620 mμ; E_{Hb620} = extinction coefficient of haemoglobin at 620 mμ; E_{SHb620} = extinction coefficient of sulphaemoglobin at 620 mμ; C_t = total haem pigment in g.%.

Blood, 0.1 ml., was pipetted into 10 ml. of a 0.1% aqueous solution of sodium carbonate, shaken, and centrifuged at 3000 r.p.m. to clear the samples. A 3.0 ml. aliquot of this sample was then placed in the spectrophotometer, and the optical density determined at 620 mμ. To the remainder of the sample, one drop of freshly prepared 5% potassium ferricyanide and one drop of 10% sodium cyanide, freshly neutralized with 12% acetic acid, were then added and the sample was thoroughly shaken and read at 578 mμ to determine the total haem pigment (C_t). The values for D_{620} and C_t were then substituted into the above formula.

(1) Twelve rabbits were injected subcutaneously with 10 mg./kg. of para-aminopropriophenone (PAPP) and 100 mg./kg. of calcium sulphide on 2 successive days. The calcium sulphide was screened through a No. 325

mesh and suspended in a concentration of 300 mg./ml. of water. The PAPP was suspended in a concentration of 10 mg./ml. of propylene glycol. Blood samples were collected at intervals from the marginal ear vein into tubes containing dried ammonium potassium oxalate mixture. The disappearance of sulphaemoglobin from the blood of these animals was followed by plotting sulphaemoglobin as a percentage of its initial level as well as the ratio D_{620}/D_{540} against the time in days after peak levels of sulphaemoglobinaemia were obtained. This latter ratio is an additional measure of sulphaemoglobin disappearance as outlined by Dod, Bierman, and Shimkin (2).

(2) Red cell survival studies were also done using radioactive sodium chromate (Cr^{51}). Three milliliters of blood from each rabbit was mixed with 1 ml. of ACD solution in a siliconized tube and 15 microcuries of Cr^{51} was added and the mixture incubated at 37° C. for 30 minutes. Fifteen milligrams of ascorbic acid was then added and the blood injected back into the rabbit. The 100% value was that taken 24 hours after the injection of the labelled cells. This was carried out on: (*a*) three normal control rabbits; (*b*) four rabbits with sulphaemoglobinaemia induced with PAPP and calcium sulphide; (*c*) five rabbits that received only PAPP (10 mg./kg.); (*d*) two rabbits that received only calcium sulphide (100 mg./kg.). In all cases the cells were labelled 24 hours after the last injection of the chemical agent used.

(3) Fifty milliliters of blood was obtained from each of three rabbits by cardiac puncture. Sulphaemoglobin was produced *in vitro* in two of these blood samples by the method of McKerns and Denstedt (5). Hydrogen sulphide was bubbled through isotonic phosphate buffer (pH 7.4) for 5 minutes. The separated red cells were mixed with an equal volume of "sulphurated buffer" for 10 minutes, centrifuged, and washed four times with 0.9% saline. The sulphurated red cells were now tagged with Cr^{51}. Each rabbit was then injected with its own sulphurated and chromium-tagged cells. Disappearance of sulphaemoglobin and radioactivity was followed according to the methods described. Fifty milliliters of blood from the third (control) animal was treated in the same fashion but for the use of an unsulphurated phosphate buffer.

Results

Fig. 1 indicates the changes in total haem pigment and sulphaemoglobin in the 12 rabbits injected with PAPP and calcium sulphide. The total haem pigment fell sharply to day 5 and then returned to normal over the next 15 days. The concentration of sulphaemoglobin reached 20% to 40% of the total haem pigment by day 3 and then disappeared in a curvilinear fashion. The ratio D_{620}/D_{540}, used as an index of sulphaemoglobin decay by Dod, Bierman, and Shimkin (2) also followed a curvilinear disappearance and the ratio returned to its preinjection value by the 45th day. This ratio is related to the absolute sulphaemoglobin value in a logarithmic fashion (6). That the disappearance of sulphaemoglobin followed a semilogarithmic

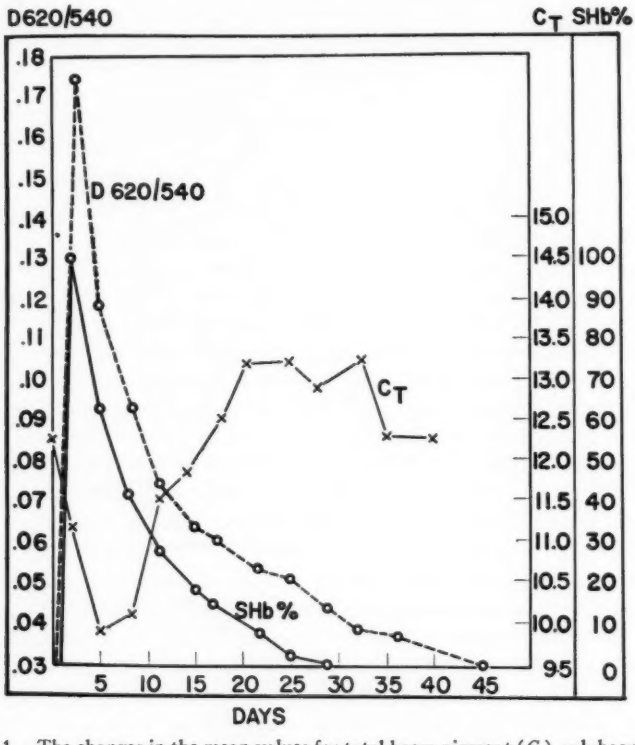


FIG. 1. The changes in the mean values for total haem pigment (C_t), sulphaemoglobin as per cent of initial value (SHb%), and D_{620}/D_{540} in 12 rabbits in which sulphaemoglobinaemia was produced by the injection of calcium sulphide and para-aminopropriophenone.

curve is shown in Fig. 2. On plotting the sulphaemoglobin decay on a semi-logarithmic scale and extrapolating the mean sulphaemoglobin level, an average red cell survival time of 36 days is obtained for the 12 rabbits.

Table I and Fig. 3 indicate the disappearance of radioactivity from the erythrocytes of three normal control rabbits tagged with Cr^{51}. This disappearance is linear with a half-life for each of the three rabbits of 16, 14, and 14 days respectively. The time taken for radioactivity to decay to a baseline of 10% is 42, 35, and 41 days. In Fig. 4 and Table I, the disappearance of radioactivity from the blood of four rabbits with sulphaemoglobinaemia is shown. The resulting half survival time of radiochromium in these rabbits was 4, 4, 6, and 3 days respectively. However, it may be noted that the time taken for radioactivity to decay to a baseline of 10% is similar in three of the animals to that for the control rabbits. Fig. 5 and Table I illustrate the disappearance of radioactivity from the blood of five rabbits which received injections of PAPP. The half-life for each of these animals was

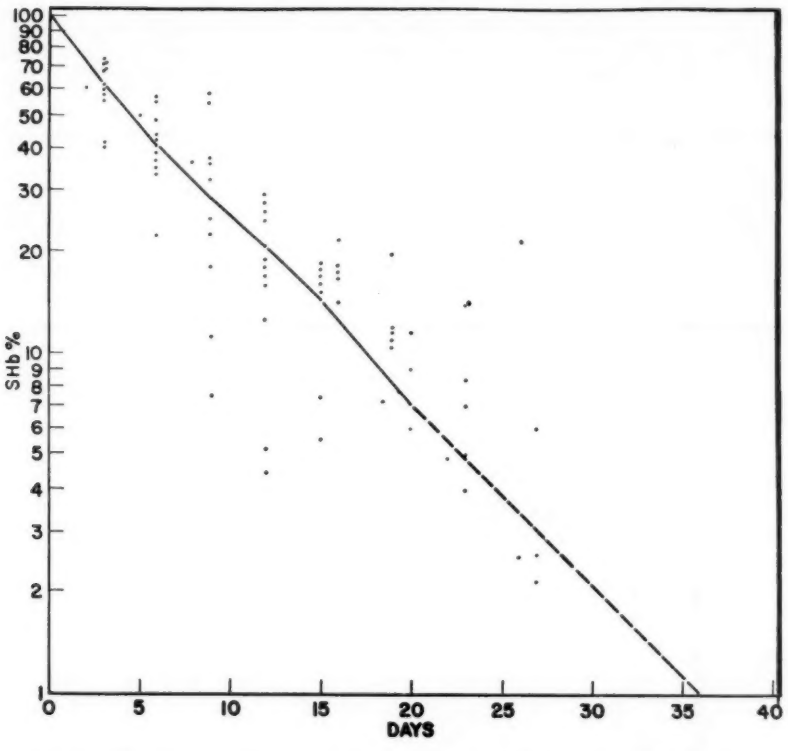


FIG. 2. The rate of disappearance of sulphaemoglobin from the blood of 12 rabbits. The logarithm of the percentage of sulphaemoglobin is plotted against the time in days. The solid line to day 20 represents the arithmetic mean of values for all animals. The dotted line represents an extrapolation to the baseline, indicating an average red cell survival of 36 days.

8, 16, 14, 7, and 8 days respectively. This suggests that a haemolytic effect is present in some of these animals. The 10% baseline was reached in 37, 47, 49, 38, and 44 days. Fig. 6 and Table I show the decay of radioactivity in two rabbits treated with calcium sulphide. Half-life for each of these animals is 17 and 18 days respectively and the time taken to reach a baseline is somewhat longer than the control animals. The difference at the 50% survival level between the normals and the sulphaemoglobin animals is highly significant ($p < .001$). There is no significant difference in 50% survival times between the normal and the PAPP group ($p > .05$) but there is a significant difference in this measurement between the PAPP and the sulphaemoglobin animals ($p = .02$).

The results of experiments in which sulphaemoglobinaemia was induced *in vitro* and the red cells tagged with Cr^{51} are shown in Fig. 7. This figure

TABLE I

THE TIME REQUIRED FOR 50% AND 90% DISAPPEARANCE OF Cr^{51} FROM THE RED CELLS OF: (*a*) NORMAL RABBITS; (*b*) RABBITS WITH SULPHAEMOGLOBINAEMIA; (*c*) RABBITS INJECTED WITH PARA-AMINOPROPRIOPHENONE (PAPP); AND (*d*) RABBITS INJECTED WITH CALCIUM SULPHIDE

Cr^{51}, % of initial activity	Time in days														
	Normal			Sulphaemoglobin				PAPP					Calcium sulphide		
50	16	14	16	4	4	6	3	8	16	14	7	8	17	18	
10	42	35	41	17	43	43	42	37	47	49	38	44	49	49	

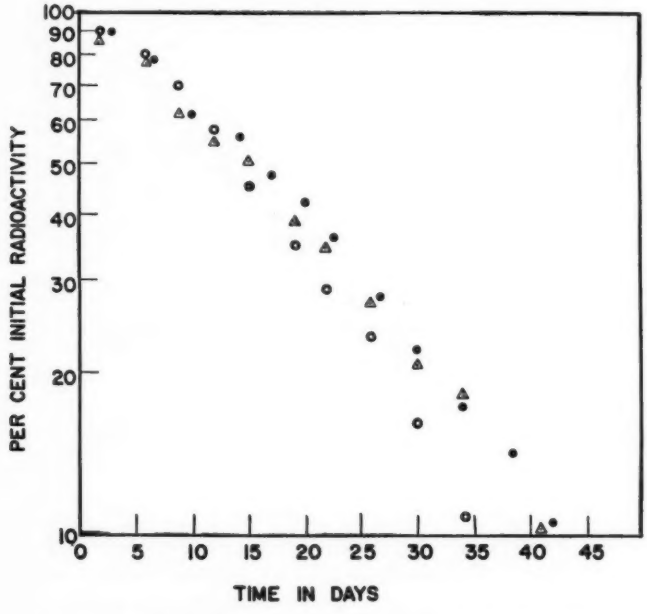


FIG. 3. The Cr^{51} red cell survival curves for three normal rabbits.

traces the disappearance of radioactivity from the circulation of two animals transfused with blood which had been tagged *in vitro* with both sulphaemoglobin and radiochromium as compared to the disappearance of radioactivity from the circulation of a rabbit whose blood had been subjected to identical treatment except for the absence of exposure to hydrogen sulphide. A considerably shortened survival of the erythrocytes tagged with sulphaemoglobin is indicated as compared to a normal survival curve for the control animal.

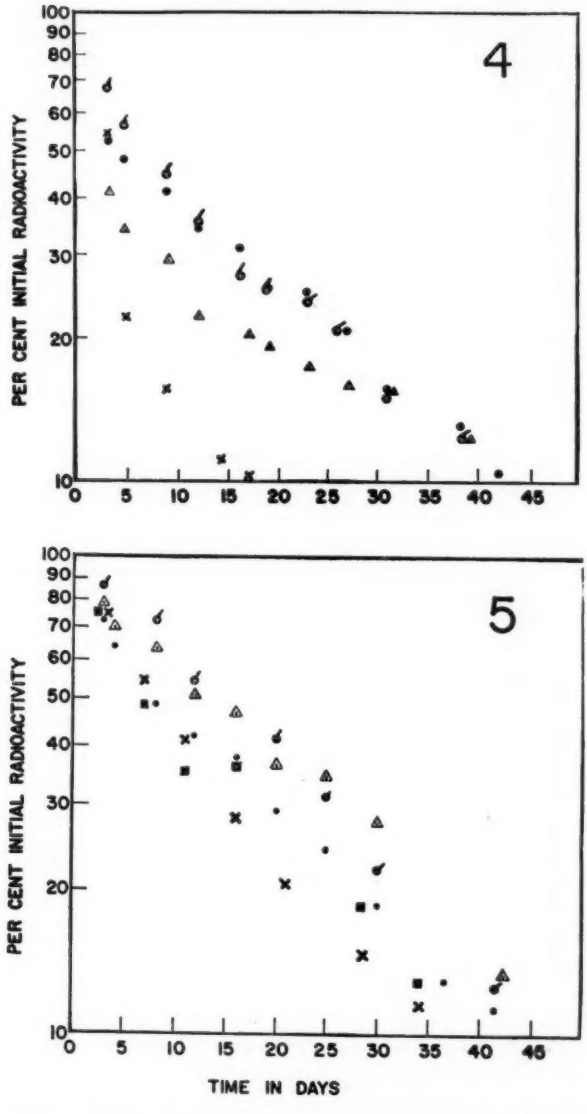
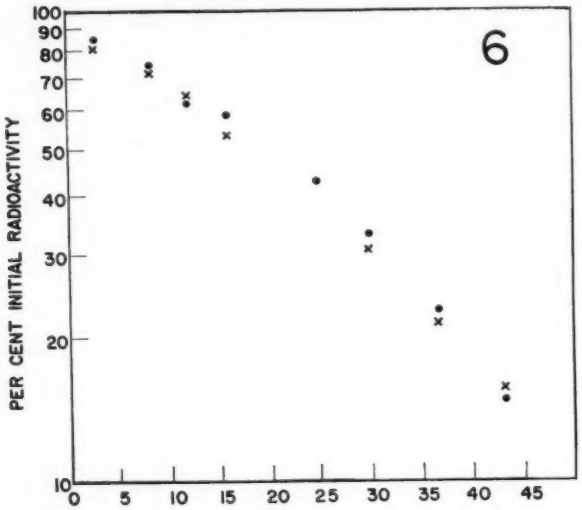


FIG. 4. The Cr^{51} red cell survival curves for four animals in which sulphaemoglobinaemia was induced by injection of calcium sulphide (100 mg./kg.) and para-aminopropriophenone (10 mg./kg.).

FIG. 5. Cr^{51} red cell survival curves for five animals injected with para-aminopropriophenone (10 mg./kg.).



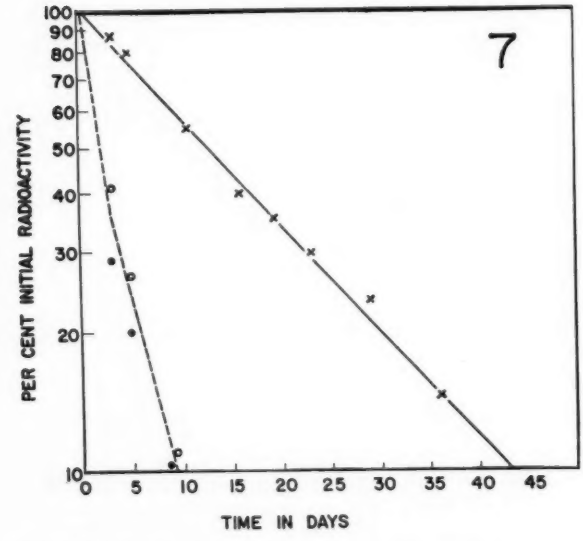


FIG. 6. The Cr^{51} red cell survival curves for two animals injected with calcium sulphide (100 mg./kg.).

FIG. 7. The Cr^{51} red cell survival curves for two rabbits in which sulphaemoglobin was produced *in vitro* (○— — —○; ●— — —●), and one rabbit in which the red cells were treated in the same manner but for the exposure to sulphurated buffer (×———×).

Discussion

The first attempt to adapt sulphaemoglobin as a red cell tag for survival studies in animals was by McKerns and Denstedt in 1949 (5). They produced sulphaemoglobinaemia in rabbits by feeding phenacetin and sulphur. They found that 20 days were required for the elimination of sulphaemoglobin from the circulating red cells following the discontinuation of the medication. They concluded that this period of time was greater than the mean life span of the rabbit erythrocyte because of the possibility of continuing formation of the sulphaemoglobin after the discontinuation of the drugs. When blood was removed from one rabbit with sulphaemoglobinaemia and transfused into a normal, the pigment persisted in the recipient animal for 11 days, which was thought to approach the true value for the rabbit red cell life span. Red blood cells in which sulphaemoglobin was induced *in vitro* by exposing them to phosphate buffer containing hydrogen sulphide survived 8 days when transfused into rabbits.

In 1951, Dod, Bierman, and Shimkin (2) induced sulphaemoglobin in rabbits by the injection of various oxidizing agents, such as phenacetin, acetanilide, sodium nitrite, and para-aminopropriophenone together with sulphur containing drugs. Using the ratio of the optical densities at wavelengths 620 and 540 as a measure of sulphaemoglobin, the disappearance of sulphaemoglobin from the blood of these animals was found to proceed in an exponential fashion, reaching a baseline in an average time of 43 days for seven animals. Using the same method of following sulphaemoglobin disappearance, we obtained a mean value of 45 days for 12 rabbits. This ratio gave a cell survival 9 days longer than the absolute determinations of sulphaemoglobin in the same animals.

Silver, Brown, and Eadie (10) produced sulphaemoglobin in dogs and rabbits by the administration of sulphur together with acetophenetidin, TNT, and PAPP. The erythrocyte life span was also followed by tagging the cells with radioactive iron. The disappearance of sulphaemoglobin from the blood of these animals followed an exponential curve and the rate of disappearance was not related to the absolute amount of sulphaemoglobin produced. They concluded that all the drugs employed as oxidizing agents produced haemolysis at a rate unrelated to sulphaemoglobin formation and that the random cell destruction was due to the oxidizing agents rather than to the presence of sulphaemoglobin in the cells.

The mean red cell survival time in normal rabbits has been estimated at 67 days using Fe^{55} as the cell tag (1) and 65 to 70 days with N^{15} (8). The Cr^{51} survival times in our control rabbits of about 40 days with a mean half-life of 15 days agree well with the data of Donahue *et al.* (3), and, allowing for chromium elution from the cell, would also approximate the findings with the other isotopes.

The results of the present group of experiments would seem to indicate that under the experimental conditions employed, sulphaemoglobin existing within the red cell does compromise its life span and leads to premature

death of the erythrocyte. Fig. 1 indicates that there is a sharp drop in total haem pigment averaging about 2 g.% which took place within 5 days of the initial injections of calcium sulphide and PAPP. The rise in haemoglobin was brisk and accompanied by reticulocytosis. Normal values had been restored by the 25th day. The concentration of sulphaemoglobin in these rabbits reached a maximum on the 3rd day and following the discontinuation of injections it declined rapidly in an exponential fashion. The semilogarithmic nature of its disappearance is evident from Fig. 2 and the extrapolated values for the 12 rabbits yield a red cell survival of 36 days —considerably shorter than the values for the other methods previously mentioned. That this is an intracorpuscular defect rather than an extracorpuscular one is shown in Fig. 7 where the sulphaemoglobin was induced *in vitro*. Here the defect is even more marked with a red cell survival of 9 days. This agrees with McKerns and Denstedt's findings of 8 days under similar circumstances. In the production of sulphaemoglobin *in vitro* there is greater trauma to the cell, but it will be noted that the control, handled in exactly the same fashion but for the exposure to hydrogen sulphide, had a normal Cr^{51} survival curve.

A similar pattern of sulphaemoglobin disappearance was obtained in 20 rats injected with PAPP (20 mg./kg.) and calcium sulphide (40 mg./kg.). In these animals the sulphaemoglobin disappeared in an exponential fashion yielding a mean red cell survival time of 16 days. In these animals the haemoglobin levels remained unchanged throughout the experimental period and no anaemia developed. The red cell survival in rats has been estimated at somewhat over 30 days using Cr^{51} (3).

The Cr^{51} survival curves shown in Figs. 3, 4, 5, and 6 lend additional support to the claim that sulphaemoglobinaemia induces haemolysis and a shortened survival time. The normal rabbits show a linear disappearance with a mean half life of 15 days and a mean 10% survival time of 39.3 days. The two rabbits injected with calcium sulphide have a mean 50% survival time of 17.5 days with a linear disappearance to 49 days, while the five rabbits injected with PAPP showed a rather irregular response with a mean half life of 10.6 days and a range of 7 to 16 days. The decay for three of the animals was curvilinear and the mean 10% value for the five animals was 43 days. The four rabbits with sulphaemoglobinaemia showed an exponential disappearance curve with a mean 50% survival of 4.5 days and a mean 10% value of 36.5 days. We would agree with Silver, Brown, and Eadie (10) that some haemolysis does attend the use of PAPP but it can be seen from the data that this is not sufficient to explain the rapid destruction of red cells sulphurated *in vivo*, and of course does not explain the rapid destruction of the cells sulphurated *in vitro* (Fig. 7).

Thus it would seem that the presence of sulphaemoglobin in the red cell does compromise the life span of the erythrocyte to some degree. In the light of these findings, it is difficult to explain Jope's initial observations of linear disappearance of sulphaemoglobin with a normal red cell survival. It is

only possible to speculate as the conditions obtaining in this series of experiments are far removed from those of Jope's observations. Jope was dealing with relatively low concentrations of sulphaemoglobin (initially 4% to 7% of the total haem pigment) in humans where the sulphaemoglobinaemia had been induced at a slower rate over a longer period of time. The possibility must be strongly considered that the sulphaemoglobinaemia-producing agent (TNT) remained in the body and continued to operate for a period of time following removal from contact. It is somewhat surprising that a normal red blood cell survival was obtained since TNT is a known haemolytic agent and haemolytic anaemia accompanied by reticulocytosis has been observed (7). Jope mentions that one of his patients had an anaemia with a haemoglobin of 70% at the beginning of the study which rose to 80% by the end of the experiment.

The absolute values obtained for the red cell life span in rabbits by the use of induced sulphaemoglobinaemia (McKerns and Denstedt—20 days; Dod, Bierman, and Shimkin—43 days; and our own findings of 36 days) would indicate that sulphaemoglobin is not a true measure of red cell life span in animals treated with sulphur compounds and an oxidizing agent. All these values are shorter than the values obtained with Fe^{55} (67 days) or N^{15} (65 to 70 days). This together with the exponential curve of disappearance and the shortened 50% survival with Cr^{51} all indicate that when sulphaemoglobin is produced by these methods there is a definite haemolytic component. It would seem that this was due to the presence of sulphaemoglobinaemia as well as to the oxidizing agents used in the production of sulphaemoglobinaemia. The change in molecular structure of haemoglobin to sulphaemoglobin may adversely influence the viability of the erythrocyte.

Acknowledgments

We are indebted to Professor F. D. White and Dr. Harold Blondal for their help and advice in this work.

References

1. Brown, I. W., Jr., and Eadie, G. S. J. Gen. Physiol. **36**, 326 (1953).
2. Dod, K. S., Bierman, H. R., and Shimkin, M. D. J. Natl. Cancer Inst. **11**, 1093 (1951).
3. Donahue, D. M., Motulsky, A. G., Giblett, E. R., Pirzio-Biroli, G., Virtanuvatti, V., and Finch, C. A. Brit. J. Haematology, **1**, 249 (1955).
4. Jope, E. M. Brit. J. Ind. Med. **3**, 136 (1946).
5. McKerns, K. W. and Denstedt, O. F. The preservation of the formed elements and of the proteins of the blood. Am. Natl. Red Cross, Boston, Mass. 1949. p. 4.
6. Michel, H. O. and Harris, J. S. J. Lab. Clin. Med. **25**, 445 (1939).
7. Minot, G. R. J. Ind. Hyg. **1**, 301 (1919).
8. Neuberger, A. and Niven, J. S. F. J. Physiol. **112**, 292 (1951).
9. Peters, J. B. and Van Slyke, D. D. Quantitative clinical estimation. Vol. II. The Williams and Wilkins Company, Baltimore, Md. 1932.
10. Silver, D., Brown, I. W., and Eadie, G. S. J. Lab. Clin. Med. **48**, 79 (1956).

CERTAIN METABOLIC EFFECTS OF CORTICOTROPIN, HYDROCORTISONE, PREDNISONE, AND ASPIRIN, IN NORMAL DOGS[1]

O. H. Gaebler, Rachel Glovinsky, Helen Lees, and Trieste Vitti[2]

Abstract

The losses of weight and nitrogen produced by daily doses of corticotropin (20 units), hydrocortisone acetate (15 mg.), and prednisone (10 mg.) were studied in normal bitches receiving a constant amount of food equal to that which before therapy had either maintained weight or permitted it to increase slowly. Loss of weight during therapy was accompanied by a decrease in the difference between water intake and urine volume, regardless of whether water intake rose or fell. Weight was restored fairly soon after cessation of therapy with prednisone, but not after corticotropin or hydrocortisone. The probability that calorigenic effects of these substances were involved is discussed. Aspirin (20 grains daily) increased nitrogen output, but weight, as well as the difference between water intake and urine volume, increased. The pattern of response to each of the compounds, was, in general, quite similar, but varied in detail in different animals or experiments. Effects of applying hydrocortisone ointment to affected areas in a bitch with dermatitis are also recorded. Prompt improvement was accompanied by losses of weight and nitrogen. Other instances in which the local and general effects of a hormone appear to be contradictory are cited.

Introduction

The purpose of this investigation was to secure, under known dietary conditions, quantitative data concerning the response of normal dogs to definite doses of corticotropin, various natural or synthetic steroids, and aspirin. Apart from their immediate interest, the data were required as control observations in experiments on depancreatized dogs treated with the same substances, and as a basis for interpreting effects of radiation in normal dogs. An additional motive was the desire to compare aspirin and newer antirheumatics with respect to their effects on metabolism of water and nitrogen.

Experimental

Normal bitches were used. The basal diet consisted of the following substances: casein, 36.2%; cracker meal, 36.2%; corn oil, 19.6%; yeast 4.0%; Phillips & Hart Salt Mixture, 4.0% (9). The diet was supplemented with 200 mg. per day of a vitamin B mixture, consisting of 750 μg. thiamine hydrochloride, 1500 μg. riboflavin, 7500 μg. nicotinic acid, 600 μg. pyridoxine, 3000 μg. calcium pantothenate, and calcium phosphate as diluent. Dog 57 received 135 g. of the basal diet daily in all experiments; Dog 64 received 150 g. daily in all experiments except the one shown in Fig. 5, in which 180 g. of basal diet, plus 25 g. of sucrose, were fed. Water present in the basal diet,

[1]Manuscript received July 31, 1957.
Contribution from the Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit, Michigan. This work was supported in part by a grant-in-aid from the Michigan Chapter, Arthritis and Rheumatism Foundation. Presented at the 20th Annual Meeting of the Canadian Physiological Society held in Montreal, October 18, 19, 1956.
[2]Predoctoral Fellow, by arrangements with The Chemistry Department of Wayne State University, Detroit, Michigan.

or derivable from it by oxidation, amounted to 58.4 ml. per 100 g. This is not included in the recorded water intake, which does, however, include water added to the food ration.

All urine periods were terminated by catheterization. Methods of animal care, as well as methods of analysis, were the same as in preceding studies (3).

The corticotropin preparation, Acthar, Gel (Armour), was given subcutaneously. Hydrocortisone acetate (Sharpe and Dohme) was given intramuscularly. Prednisone (Schering), which is $\Delta^{1,4}$ pregnadiene-17 alpha, 21-diol-3,11-20-trione, and aspirin were given by mouth in the doses indicated on the figures. Aspirin was powdered and stirred into the food. It could not be given in this way in doses larger than 20 grains without causing vomiting or refusal of food. In one experiment, 2.5 mg. of growth hormone (Armour No. M208) was injected subcutaneously daily for 6 days.

Results

That growth hormone and corticotropin have opposite effects is shown graphically in Fig. 1. Administration of the former is followed by diminished nitrogen output, an increase in water intake exceeding the rise in urine volume, and consequent gain in weight. Injection of corticotropin is followed by a marked rise in nitrogen output. Water intake rises, but urine volume approaches it more closely. Marked loss in weight occurs, and while losses stop when the treatment is discontinued, the loss in weight is not restored. Unfortunately, circumstances did not permit observations on energy metabolism, such as those reported in earlier experiments (2).

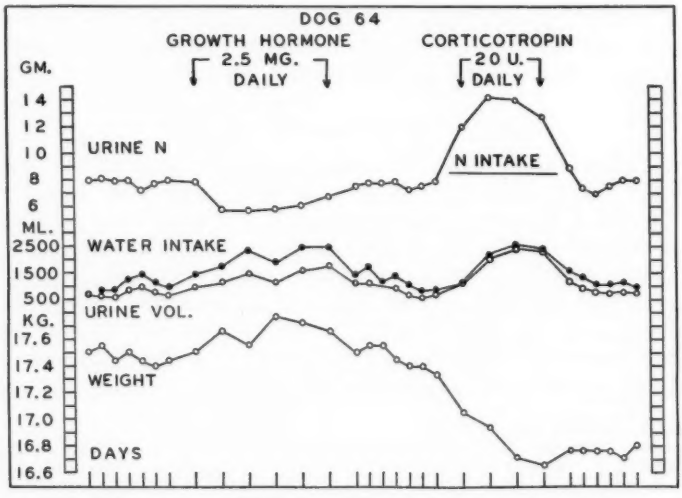
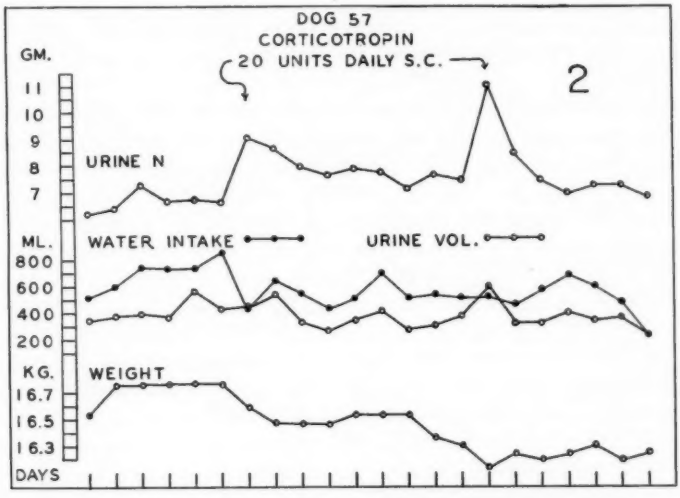


FIG. 1. Experiments showing that growth hormone and corticotropin have opposite effects on water balance as well as on nitrogen output. Marks on the time scale, irrespective of the distance between them, indicate days.

When corticotropin was given for a longer period to another animal, the findings were somewhat different (Fig. 2). The rise in nitrogen output was transient, as in the previous experiment, but a large loss occurred on the last day of injection. Diminution in the excess of water intake over urine volume



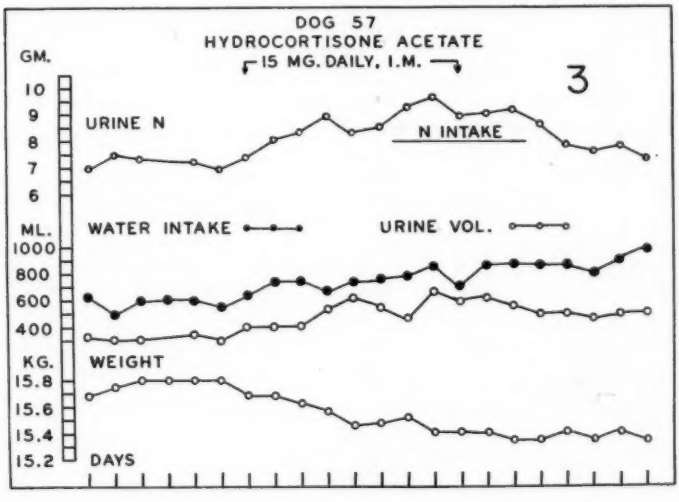


FIG. 2. Effects of corticotropin on urine nitrogen, water intake, urine volume, and weight.

FIG. 3. Effects of hydrocortisone acetate, administered intramuscularly. The indicated level of nitrogen intake applies to the entire experiment.

was not great, and occurred because water intake fell. Marked loss of weight, without early restoration, was a constant finding in these two quite different animals. Dog 64 (Fig. 1) is very high strung, while Dog 57 (Fig. 2) is extremely placid.

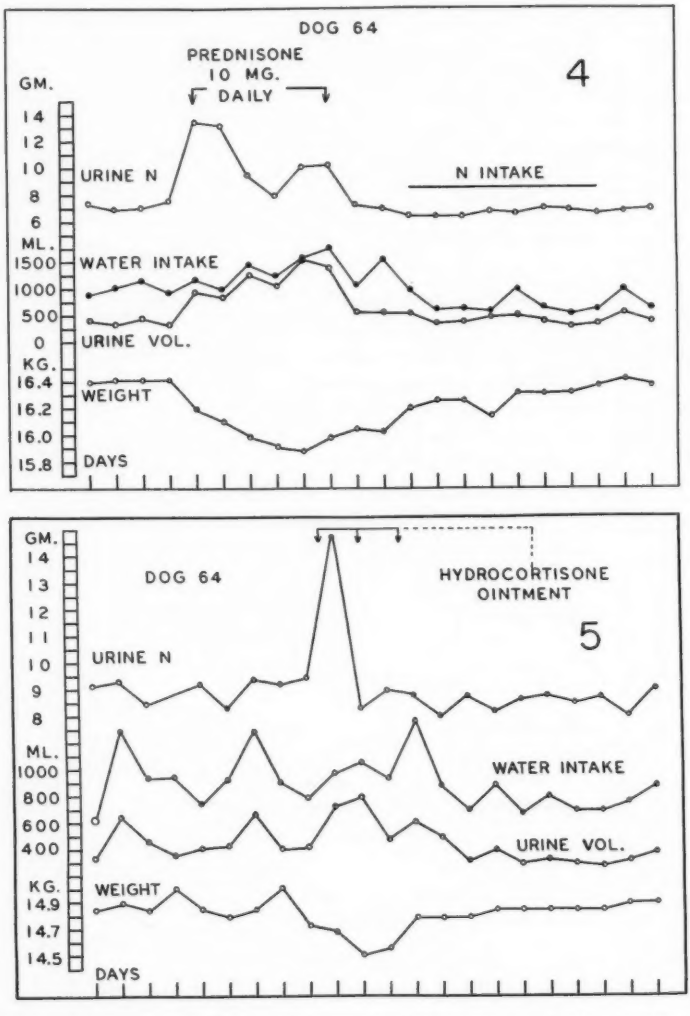


FIG. 4. An experiment with prednisone, showing return to previous weight, after marked losses of nitrogen and weight.

FIG. 5. Nitrogen loss, decrease of the difference between water intake and urine volume, and loss of weight, occurred in a dog with dermatitis, during successful treatment with hydrocortisone ointment.

Results with hydrocortisone acetate (Fig. 3) were similar to those with corticotropin, but the rise in nitrogen output was sustained—in fact, increased—during therapy. A decrease in the difference between water intake and urine volume, and unrestored loss of weight, were again observed.

During the first 4 days, the effect of prednisone (Fig. 4) on nitrogen output was like that of corticotropin in the same animal, but a secondary rise occurred later. The approach of urine volume to water intake during therapy was very evident, but restoration of weight began promptly after cessation of medication.

The high-strung Dog 64 occasionally had episodes of dermatitis, affecting paws, belly, flank, and snout. During one of these recurring attacks, hydrocortisone ointment (1%) was applied to all affected parts. The animal was not muzzled, since the snout was involved, but did not, during observation, lick off the ointment, of which 20 g., containing 200 mg. of hydrocortisone, was applied in 3 days. Very great outpouring of nitrogen, approach of urine volume to water intake, and loss in weight, all occurred on the first 2 days of treatment (Fig. 5), during which there was marked improvement of the dermatitis. The weight loss was transient. Whether or not absorption of hydrocortisone was entirely percutaneous in this case, percutaneous absorption of another steroid has been established by Livingood and co-workers (6).

The effects of aspirin (Table I) were clearly different from those obtained with corticotropin, hydrocortisone, and prednisone. Nitrogen output increased, but the difference between water intake and urine volume, as well as the weight, increased. The weight of Dog 57 was rising when it became necessary to discontinue aspirin.

TABLE I

EFFECTS OF ASPIRIN ON WATER BALANCE AND NITROGEN OUTPUT

Experiment	Start of period	Duration, days	Water intake, ml./day	Urine vol., ml./day	Weight, kg.	Urine N, g./day
Dog 64						
Control	5–20–56	8	781	512	16.49	7.01
Expt.*	5–28–56	6	844	385	16.64	7.82
Terminal	6– 3–56	8	664	396	16.37	6.70
Dog 57						
Control	8–27–56	6	470	282	16.26	7.30
Expt.*	9– 2–56	2	660	360	16.25	8.00
Terminal	9– 6–56	6	462	315	16.02	6.04

*20 grains aspirin per day.

Discussion

It is generally known that corticotropin, hydrocortisone, and related natural or synthetic steroids cause loss of weight and nitrogen. Maximal therapeutic effects, with minimal "catabolic" ones, constitute one of the objectives in synthesis of new steroids. It is evident, however, that losses of weight and

nitrogen (Fig. 5), which would certainly be considered catabolic, were accompanied by prompt healing of dermatitis. Reduction of edema of the skin may be a partial explanation. In any event, effects of a hormone at a given site do not necessarily conform with its over-all effect. This point has been emphasized previously, in studies with growth hormone (4, 11) which has well-known anabolic effects. The problem of defining growth, anabolism, and catabolism, has been admirably presented by Weiss (10).

Meites (7) reported that, in immature rats fed a B_{12} deficient diet ad libitum, the unfavorable effect of cortisone (0.5 mg. daily) on body weight was partly overcome by addition of B_{12} or aureomycin to the diet. The beneficial effect of these supplements was accompanied by an increase in food intake. When food intake is restricted, vitamin B_{12} does not counteract the effect of cortisone on body weight (8). Effects of cortisone on the requirement for B_{12}, pyridoxin, and other vitamins have been investigated in the same laboratory.

In the present experiments on adult bitches, food intake was constant, having been adjusted so that the daily ration was completely consumed, and that body weight, before therapy, was maintained or slowly increased. When reversal of weight loss was delayed long after cessation of therapy, increase in food intake readily restored body weight. Apparently the caloric requirement was altered. Evans, Contopoulos, and Simpson (1) have shown that the calorigenic effect of corticotropin occurs indirectly, by way of adrenal steroid production. Further studies with individual steroids should, therefore, suffice for elucidation of the problem of weight loss.

In connection with the prolonged effect of corticotropin and steroids, it may be of interest to recall the finding of Levine (5) that in premature infants who were lethargic improvement following corticotropin persisted after treatment was discontinued.

Acknowledgment

The authors wish to thank the Michigan Chapter, Arthritis and Rheumatism Foundation, for a generous grant-in-aid in support of this study.

References

1. Evans, E. S., Contopoulos, A. N., and Simpson, M. E. Endocrinology, **60**, 403 (1957).
2. Gaebler, O. H. J. Exptl. Med. **57**, 349 (1933); Am. J. Physiol. **110**, 584 (1935).
3. Gaebler, O. H., Bartlett, P. D., and Sweeney, M. J. Am. J. Physiol. **165**, 486 (1951).
4. Gaebler, O. H. and Mathies, J. C. Endocrinology, **48**, 623 (1951).
5. Levine, S. Z. Infant metabolism. MacMillan, New York. 1956. p. 399.
6. Livingood, C. S., Hildebrand, J. F., Key, J. S., and Smith, R. W., Jr. A. M. A. Arch. Dermatol. **72**, 313 (1955).
7. Meites, J. Proc. Soc. Exptl. Biol. Med. **78**, 692 (1951).
8. Meites, J. Private communication.
9. Phillips, P. H. and Hart, E. B. J. Biol. Chem. **109**, 657 (1935).
10. Weiss, P. What is growth? *In* Hypophyseal growth hormone, nature and actions. *Edited by* R. W. Smith, O. H. Gaebler, and C. H. N. Long. The Blakiston Division, McGraw-Hill Book Company, Inc., New York. 1955. p. 3.
11. Zuchlewski, A. C. and Gaebler, O. H. Arch. Biochem. Biophys. **66**, 463 (1957).

ON THE INCORPORATION OF LABELLED ADENINE INTO THE PURINES OF THE NUCLEIC ACIDS IN THE RAT[1]

R. V. TOMLINSON AND S. H. ZBARSKY

Abstract

Adenine labelled with C^{14} in position 2 and with N^{15} in positions 1 and 3 was injected intraperitoneally into rats, and the purines of the nucleic acids from the pooled viscera were isolated. The C^{14}/N^{15} ratios of the isolated adenine and guanine were considerably lower than that of the injected adenine, indicating that during the formation of the nucleic acid purines from the administered material there was a loss of C^{14} relative to N^{15}. This loss was larger for the guanine, which had a C^{14}/N^{15} ratio 52–67% lower than that of the injected adenine. Metabolic removal of carbon 2 of the adenine was shown further by the excretion of 22% of the injected radioactivity as respiratory carbon dioxide. Fifty-three per cent of the administered radioactivity was excreted in the urine and of this less than half was accounted for as allantoin, adenine, and urea. The C^{14}/N^{15} ratio of the urinary allantoin was intermediate in value between those of the adenine and guanine. The evidence obtained indicates that during the metabolic conversion of adenine to guanine the purine ring may undergo rupture at the 2 position. A possible mechanism for the reaction is presented.

Introduction

In a previous paper from this laboratory (14) it was reported that when 2-C^{14}-adenine was administered to rats by intraperitoneal injection, 8.5–9.4% of the radioactivity was excreted in the expired carbon dioxide. Consideration of other experimental evidence pointing to the metabolic stability of the purine ring and its derivatives in the rat led to the conclusion that the $C^{14}O_2$ did not arise through the total degradation of a portion of the injected adenine (14). It was felt that the observed results might be explained by assuming a conversion of the 2-C^{14}-adenine to 2-C^{14}-inosinic acid followed by a formate exchange reaction as described by Buchanan and Schulman (6). Such a reaction could lead to the formation of a derivative of carbon 2 which is oxidized to carbon dioxide.

The finding of radioactivity in the expired air of rats receiving 2-C^{14}-adenine is in contrast with the results obtained by Marrian *et al.* (9), who fed 8-C^{14}-adenine to rats and found no significant production of $C^{14}O_2$. Further, these authors observed that on feeding to rats a mixture of 1,3-N^{15}-adenine and 8-C^{14}-adenine there was a "parallel incorporation of the N^{15} and C^{14} into each of the adenylic and guanylic acid isomers obtained from the visceral nucleic acids" (9). This and related results reviewed by Brown and Roll (5) have been taken to indicate that in the conversion of administered adenine to guanine of the tissue nucleic acids, the purine ring remains intact. None of the experiments cited, however, was carried out with 2-labelled adenine and the possibility still remained that there might be a loss of the carbon

[1] Manuscript received July 2, 1957.
Contribution from the Department of Biochemistry, University of British Columbia, Vancouver 8, B.C. Taken from a thesis presented by R. V. Tomlinson to the Faculty of Graduate Studies, University of British Columbia, in partial fulfillment of the requirements for the degree of Master of Science.

in position 2 of adenine *relative* to the remainder of the purine ring. In this connection Abrams recently reported (1) that when 2-C^{13}-8-C^{14}-adenine was injected into rats there was no significant exchange of carbon 2 relative to carbon 8 during the incorporation of the adenine into liver ribonucleic acid. No examination was made of the expired carbon dioxide.

The finding of $C^{14}O_2$ in the expired air of rats injected with 2-C^{14}-adenine (14) led to the present study with adenine labelled with N^{15} in nitrogens 1 and 3 and with C^{14} in carbon 2. It was believed that a comparison of the C^{14}/N^{15} ratio of injected doubly-labelled adenine with those of the isolated adenine and guanine would indicate whether a difference existed in the metabolic lability of carbon 2 relative to the remainder of the purine ring. Rats injected with the doubly-labelled adenine expired as $C^{14}O_2$ approximately 22% of the radioactivity. The C^{14}/N^{15} ratios of the tissue purines were lower than that of the injected adenine, the ratio in the guanine in one experiment being reduced by 52%. These results provided further evidence that position 2 of the ring is metabolically more reactive than the remainder of the molecule.

Methods

Radioactivity Measurements

Unless otherwise stated, all materials were converted to barium carbonate and counted as "infinitely thick" samples using a windowless gas flow counter (14, 15). Sufficient counts were recorded for each sample to give a counting error of 5%.

N^{15} Analyses

Samples of N_2 gas were obtained from the materials analyzed by the method of Rittenberg (11). The N^{15} contents of the gas samples were determined by Dr. Harry J. Svec, Department of Chemistry, Iowa State College.

Preparation of 1,3-N^{15}-2-C^{14}-Adenine

1,3-N^{15}-Adenine was synthesized as described by Cavalieri, Tinker, and Bendich (8). 2-C^{14}-Adenine was prepared by the procedure of Paterson and Zbarsky (10). The doubly-labelled purine was obtained by dissolving portions of each of the above compounds in dilute hydrochloric acid solution and reisolating the adenine hydrochloride. The purity of the material was established by ultraviolet spectrophotometry, paper chromatography, and comparison of the decomposition point of its picrate derivative with that prepared from authentic adenine. Radioautography (15) indicated the absence of detectable amounts of radioactive contaminants. The doubly-labelled adenine had a specific activity of 2.24×10^6 counts/minute/millimole and a N^{15} content of 2.997 atom % excess.

Metabolism Experiments

Groups of male rats of the Wistar strain from the colony at the University of British Columbia were used for these experiments. The doubly-labelled adenine was dissolved in phosphate buffer, pH 3.8, and injected intraperitoneally. In Experiment 1, three rats with a combined weight of 515 g. were

used. Each animal received per injection 8.62 mg. of adenine in 0.9 ml. of solution. Injections were administered at 12 hour intervals, the dose of adenine being equivalent to 100 mg. per kilogram body weight per day. This experiment was terminated after 24 hours. In this experiment a total of 8.77×10^5 counts per minute of C^{14} was injected. In Experiment 2, two rats with a combined weight of 462 g. were used and the dose of adenine was reduced to 50 mg. per kilogram body weight per day because the animals receiving the higher doses in the first experiment exhibited toxic symptoms. Each rat received 5.8 mg. of adenine per injection in 0.62 ml. of solution. This experiment was continued for 96 hours, a total of 1.57×10^6 counts per minute of C^{14} being administered.

As described in the previous paper (14), at the termination of the experiments, the animals were killed, the viscera excised, pooled, and homogenized. The acid-soluble nucleotides were extracted with three 100 ml. portions of cold 5% trichloroacetic solution. From the tissue residue the nucleic acids were isolated and hydrolyzed to yield the purines which were estimated quantitatively (14) and analyzed for N^{15} and C^{14} content. The acid-soluble extracts were combined and the trichloroacetic acid removed by extraction with ether. The pale yellow solution was concentrated to a volume of 10 ml. by distillation under reduced pressure, made 1 *N* in hydrochloric acid, and hydrolyzed by heating at 100° C. for 1 hour. The hydrolyzate was treated as described later.

The treatment of the expired carbon dioxide and urine have been described previously (14,15). Analyses for C^{14} and N^{15} were carried out on urinary urea and on allantoin isolated from the pooled urine collected in Experiment 2 (4). The radioactivity present in the urine as adenine was determined using the carrier technique (14).

Results

Purines of the Visceral Nucleic Acids

The isotope analyses of the purines obtained on hydrolysis of the visceral nucleic acids are presented in Table I. Of particular significance are the figures shown in the last column where the C^{14}/N^{15} ratios of the isolated purines are compared with that of the injected adenine. These data demonstrate that in the processes by which injected adenine was incorporated into the purines of the nucleic acids there was a loss of C^{14} relative to N^{15}. The loss of C^{14} was particularly striking in the case of the guanine. It can be concluded from the results that in the metabolic conversion of adenine to guanine by the rat, the purine ring does not remain intact and that the carbon in position 2 of the nucleic acid guanine represents only part of the carbon present in that position in the injected adenine.

The lowered C^{14}/N^{15} ratio in the excreted allantoin provides further evidence of replacement of carbon 2 relative to the nitrogens in positions 1 and 3. The fact that the allantoin ratio fell between those of the adenine and guanine is possibly a result of its being derived from both purines.

TABLE I

ISOTOPE CONTENT OF ADENINE AND GUANINE FROM VISCERAL NUCLEIC ACIDS OF RATS GIVEN 1,3-N^{15}-2-C^{14}-ADENINE INTRAPERITONEALLY

Purine	Total C^{14}, c.p.m. $\times 10^{-6}$	Specific activity, c.p.m./millimole $\times 10^{-4}$ (*a*)	Atom % excess N^{15} (*b*)	C^{14}/N^{15} (*a*/*b*)
Expt. 1				
Injected adenine	8.6	224.0	2.997	1.000*
Isolated adenine	0.172	10.06	0.163	0.824
Isolated guanine	0.080	2.64	0.106	0.333
Expt. 2				
Injected adenine	15.7	224.0	2.997	1.000*
Isolated adenine	0.128	21.26	0.336	0.847
Isolated guanine	0.103	6.01	0.168	0.479
Urinary allantoin	2.58	11.98	0.223	0.719

*These ratios have been calculated on the basis of a ratio of 1.000 for the injected adenine.

Acid-Soluble Purines

The purines of the hydrolyzate of the acid-soluble extract were isolated by ion-exchange chromatography on Dowex-50 resin, 200–400 mesh, H^+ form, elution being carried out with 1 *N* hydrochloric acid. Three main ultraviolet-absorbing fractions of eluate were obtained. A representative portion of each fraction was concentrated to 0.25 ml. and this was applied to a paper chromatogram which was developed with Wyatt's solvent (13). The first fraction contained a mixture of pyrimidine compounds and hypoxanthine, and the second and third contained guanine and adenine respectively. Radioactive areas on the paper chromatograms were located by scanning with an automatic windowless gas flow scanner* and found to correspond with the areas occupied by the purines. Disks were punched out of these areas and assayed for radioactivity. The disks were then extracted for 8 hours with 0.1 *N* hydrochloric acid and the identity and concentration of the purine in each extract were determined by ultraviolet spectrophotometry. From these data the relative specific activities of the purines in the acid-soluble extract were calculated and are presented in Table II. The amounts of purines isolated from the acid-soluble fraction were too small to permit N^{15}

TABLE II

RELATIVE SPECIFIC ACTIVITIES OF ADENINE, HYPOXANTHINE, AND GUANINE IN THE ACID-SOLUBLE EXTRACT OF VISCERAL TISSUE OF RATS RECEIVING 1,3-N^{15}-2-C^{14}-ADENINE INTRAPERITONEALLY

Expt. No.	Adenine	Hypoxanthine	Guanine
1	1.00	0.97	0.29
2	1.00	0.92	0.24

* This apparatus was designed and built by Mr. F. N. McGillivary, formerly of the British Columbia Medical Research Institute, Vancouver, B.C.

analyses to be made so that it was not possible to compare C^{14}/N^{15} ratios as was done for the nucleic acid purines. It can be noted that the specific activity of the hypoxanthine was very close to that of the adenine whereas that of the guanine was considerably lower. If it is assumed that the hypoxanthine was derived from adenine or its derivatives in the acid-soluble pool, the results suggest that there is a very small amount of hypoxanthine or its nucleotides or that the hypoxanthine pool turns over very rapidly. The lower specific activity of the guanine is compatible with the hypothesis that the original carbon 2 of adenine is partly lost and replaced during conversion to guanine. Other possible explanations which should perhaps be considered are that there is a low rate of turnover of the acid-soluble guanine pool or that there is a considerable dilution of the radioactive guanine (or its nucleotides) with guanine compounds in the acid-soluble fraction. With regard to the latter point, observations of a qualitative nature in the authors' laboratory indicate that the guanine content of the nucleotide fraction of the viscera is of an order that would tend to suggest that dilution by non-radioactive guanine is a minor factor in lowering the specific activity of the acid-soluble guanine. No conclusions can be made, however, until quantitative data are available.

Expired Carbon Dioxide

In Experiment 1, expired CO_2 was collected for the first 12 hours only. During this time 60,000 counts per minute of $C^{14}O_2$ were excreted, representing 14% of the radioactivity in the first injection of adenine. The excretion of C^{14} in the respiratory CO_2 in Experiment 2 is shown in Table III. The total radioactivity in the expired CO_2 accounted for 22% of that injected as adenine. This amount is higher than that reported in the experiments of Zbarsky and Paterson (14) in which 8.5–9.4% of the C^{14} of injected 2-C^{14}-adenine was excreted as CO_2. However, in the latter experiments the dose of adenine administered was somewhat lower than that used in the present work. An interesting observation was the diurnal variation in excretion of $C^{14}O_2$.

TABLE III

C^{14} CONTENT OF CARBON DIOXIDE EXPIRED BY RATS RECEIVING 1,3-N^{15}-2-C^{14}-ADENINE BY INTRAPERITONEAL INJECTION (1.57×10^6 C.P.M.)

Collection period, hr.	Total c.p.m. $\times 10^{-4}$	Specific activity, c.p.m./mg. C
0–12	4.40	11.39
12–24	3.37	19.25
24–36	4.37	11.25
36–48	7.03	17.80
48–60	3.27	8.90
60–72	4.47	12.65
72–84	3.40	8.80
84–96	4.17	10.41
Total	34.48×10^4	

The CO_2 excreted overnight had a higher total radioactivity and a higher specific activity than that excreted during the day. The results with respect to the excretion of radioactive carbon dioxide by rats injected with adenine labelled with C^{14} in the 2-position are in accord with the finding of a reduced C^{14}/N^{15} ratio in the nucleic acid purines.

Urine

In the first experiment 49.5% of the injected radioactivity was excreted in the urine. Of this, 1% was present as urea, which contained 0.009 atom % excess N^{15}. The excretion of C^{14} in the urine and the C^{14} and N^{15} content of the urea in experiment 2 are shown in Table IV. The radioactivity in

TABLE IV

EXCRETION OF C^{14} AND N^{15} IN THE URINE OF RATS GIVEN INTRAPERITONEAL INJECTION OF 1,3-N^{15}-2-C^{14}-ADENINE (1.57×10^6 C.P.M.)

Whole urine			Urea			
Period of collection, hr.	Total C^{14}, c.p.m. $\times 10^{-4}$	Spec. act., c.p.m. /mg. C	Period of collection, hr.	Total C^{14}, c.p.m. $\times 10^{-3}$	Spec. act., c.p.m. /mg. C	N^{15}, atom % excess
0–12	6.94	231				
12–24	6.71	266	0–24	5.44	35.05	0.014
24–36	10.99	384				
36–48	11.57	303	24–48	1.02	17.20	0.014
48–60	12.91	461				
60–72	13.16	376	48–72	1.72	10.20	0.012
72–84	11.67	459				
84–96	9.37	205	72–96	1.20	8.80	0.014
Total	83.32×10^4			9.38×10^3		

the urine represented 53% of that injected. The total daily excretion of C^{14} and the specific activity of the carbon rose during the first 3 days and then declined. The specific radioactivity of the urea was highest on the first day and declined gradually during the experiment. The N^{15} content of the urea, which was low, remained constant.

A large fraction of the radioactivity in the urine was present as allantoin. On the basis of total allantoin determinations and the radioactivity present in a crystalline sample isolated from the pooled urine, it was calculated that the urinary allantoin accounted for 2.57×10^5 counts per minute or 31% of the C^{14} in the urine. Adenine was isolated from a portion of the urine after adding non-radioactive carrier and was found to contain 12% of the urinary C^{14}.

Discussion

The results of the experiments which have been described indicate that when injected 1,3-N^{15}-2-C^{14}-adenine is converted to nucleic acid adenine and guanine in the rat, there is a loss of the C^{14} relative to the N^{15}. The C^{14}/N^{15} ratio of the nucleic acid adenine was 16% less and that of the guanine

52–67% less than that of the injected adenine. It appears reasonable to conclude, therefore, that the adenine undergoes metabolic reactions whereby the carbon in position 2 is reversibly removed and replaced, while the rest of the molecule presumably remains intact. These conclusions are supported by the finding of 22% of the radioactivity in the expired carbon dioxide, and a very small amount of N^{15} in the urinary urea. The latter observation indicates a very low degree of complete metabolic degradation of the adenine.

To account for the decrease in the C^{14}/N^{15} ratios of the nucleic acid guanine, two major possibilities may be considered.

1. The conversion of adenine to guanine may take place by more than one pathway. One might involve no rupture of the purine ring and would yield guanine with the same C^{14}/N^{15} ratio as the administered adenine. A second pathway might involve complete replacement of carbon 2 by non-isotopic carbon, leaving the 1 and 3 nitrogens intact. The over-all result of these reactions would be guanine with a reduced C^{14}/N^{15} ratio.

2. A reversible removal of carbon 2 to form an intermediate which would supply carbon for reincorporation into the purine ring. Since this intermediate would receive carbon from non-radioactive adenine as well, its radioactivity would be diluted, thereby lowering the amount reincorporated into the labelled purine and yielding guanine and adenine with a reduced C^{14} content relative to the N^{15}.

Using bone marrow extracts, Abrams and Bentley (2) showed that when adenine is converted to guanine the purine is first bound to ribose and the adenosine thus formed passes through inosine before guanine is derived. It was further demonstrated that adenosine-5′-phosphate, acting as a guanine precursor, goes through a hypoxanthine stage to some extent. In a later paper (3) these authors demonstrated that inosinic acid can be transformed to adenylic and guanylic acids by a soluble enzyme system from rabbit bone marrow. The conversion of adenine to guanine presumably involves, therefore, the following reaction sequence: adenine → adenosine-5′-phosphate → inosinic acid → xanthosine-5′-phosphate → guanosine-5′-phosphate. Assuming that inosinic acid is indeed an intermediate in the conversion of adenine to guanine, an explanation for the loss of radioactivity from 2-labelled adenine might be found if there is present in mammalian tissue an inosinic transformylase system similar to that described for pigeon liver by Buchanan and Schulman (6) and discussed further by Buchanan *et al.* (7). By this exchange system the reactions involved in the present experiments would be cleavage of the purine ring, equilibration of the reaction products with *non*-radioactive formate from other metabolic pathways, and re-formation of the purine ring by incorporation in the 2-position of formate with a reduced specific activity. Metabolic oxidation of part of the reaction products could account for the appearance of C^{14} in the expired carbon dioxide.

Recently Warren (12) has described transformylase obtained from chicken liver which will catalyze the reaction inosinic acid + tetrahydrofolic acid → 4-amino-5-imidazole carboxamide ribotide + formyltetrahydrofolic acid,

implicating the latter as a carrier of the formyl group in the exchange reaction. It would be of considerable interest to determine whether similar systems occur in mammalian tissue because the presence of such systems would assist in explaining the observations in the present work. No explanation is available to account for the differences between the findings presented herein and those obtained by Abrams (1) with 2-C^{13}-8-C^{14}-adenine.

Acknowledgments

This work was supported by grants from the National Research Council of Canada. The authors are indebted to Dr. A. R. P. Paterson for many helpful discussions during the preparation of the manuscript.

References

1. Abrams, R. Biochim. et Biophys. Acta, **21**, 439 (1956).
2. Abrams, R. and Bentley, M. Arch. Biochem. Biophys. **56**, 184 (1955).
3. Abrams, R. and Bentley, M. J. Am. Chem. Soc. **77**, 4179 (1955).
4. Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F. J. Biol. Chem. **172**, 469 (1948).
5. Brown, G. B. and Roll, P. M. *In* Nucleic acids. Vol. II. *Edited by* E. Chargaff and J. N. Davidson. Academic Press, Inc., New York. 1955.
6. Buchanan, J. M. and Schulman, M. P. J. Biol. Chem. **202**, 241 (1953).
7. Buchanan, J. M., Levenberg, B., Flaks, J. G., and Gladner, J. A. *In* A symposium on amino acid metabolism. *Edited by* W. O. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md. 1955.
8. Cavalieri, L. F., Tinker, J. F., and Bendich, A. J. Am. Chem. Soc. **71**, 533 (1949).
9. Marrian, D. H., Spicer, V. L., Balis, M. E., and Brown, G. B. J. Biol. Chem. **189**, 533 (1951).
10. Paterson, A. R. P. and Zbarsky, S. H. J. Am. Chem. Soc. **75**, 5753 (1953).
11. Rittenberg, D. Preparation and measurement of isotopic tracers. University of Michigan Press, Ann Arbor, Michigan. 1946.
12. Warren, L. Federation Proc. **16**, 267 (1957).
13. Wyatt, G. R. Biochem. J. **48**, 584 (1951).
14. Zbarsky, S. H. and Paterson, A. R. P. Can. J. Biochem. and Physiol. **35**, 55 (1957).
15. Zbarsky, S. H. and Wright, W. D. Can. J. Med. Sci. **31**, 151 (1953).

FURTHER STUDIES ON THE RELATIONSHIP OF GLUCAGON TO THE ALPHA CELL OF THE PANCREAS[1]

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Abstract

The relationship of the morphology of the alpha cell and the amount of glucagon present in the pancreas of dogs and rabbits treated with drugs reputed to be toxic for the alpha cell has been further investigated. A summary is given of several experiments dealing with the lack of alpha cell damage in several species following the administration of drugs reputed to be toxic for the alpha cell. It is concluded that glucagon is produced by the alpha cell of the pancreas, and that much of the confusion on this subject probably results from a failure to recognize the technical difficulties involved in obtaining accurate morphological definition of the structure of the alpha cell at the time the tissue was excised from the body.

Introduction

From recent work we have presented evidence suggesting that glucagon is intimately related to the alpha cells of the pancreas (2, 5, 6, 7, 8) but there are still many differences of opinion on this subject. Because of this we believe it would be timely to report our observations relating glucagon content of the pancreas to the morphology of the alpha cells in a number of animal species subjected to different drugs toxic to the alpha cell.

Methods and Results

A total of 15 dogs and 55 rabbits were used. Healthy mongrel dogs weighing between 20 and 40 lb. were fed raw tripe; New Zealand White rabbits weighing between 2.5 and 5.5 kg. were fed Purina Rabbit Chow; all animals had free access to water.

Alloxan, when used, was injected as a 5% solution intravenously, 75 mg./kg. for dogs and 150 mg. /kg. for rabbits; synthalin A* was injected as a single subcutaneous injection, using a 1% solution. Blood glucose was determined by a micromodification of the Folin–Wu method (21).

Animals were killed with an overdose of nembutal. In dogs, glucagon extracts were obtained separately from the uncinate process and the remainder of the pancreas. As in similar types of experiments, sections were taken from the uncinate process and the tail of the pancreas in order to confirm the absence of alpha cells in the uncinate process of the pancreas (6). In rabbits, a section from the splenic end of the pancreas was used for histological examination while glucagon was extracted from the remainder of the pancreas. Tissues were fixed in Zenker formol. Routine sections cut at 2.5 microns were stained with a modification of Masson's trichrome (2, 3), Gomori's chrome alum hematoxylin (3), and with Gomori's aldehyde fuchsin stains (20).

[1]Manuscript received July 8, 1957.
Contribution from the Department of Pathology of Queen's University, Kingston, Ontario. This work was supported by grants from the National Research Council of Canada, The Canadian Life Insurance Officers Association, and the Banting Research Foundation.
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Glucagon was extracted following the procedure of Best *et al.* (9) and tested according to the method of Staub and Behrens (23). The details of these procedures, as applied in this laboratory, have been given elsewhere (6).

(Group 1) Rabbit Control

The pancreas of four untreated rabbits were used as controls. Individual extracts from 0.5 g. of pancreas were tested once, after which the remainder of these four extracts was pooled and the equivalent of 1 g. of pancreas tested in triplicate.

Detailed description of the cytology of the alpha and delta cells in the rabbit is to be found in earlier reports (1, 2, 3, 4).

When tested, the pancreatic extracts of the control rabbits elicited the expected hyperglycemia. This was slightly higher when the equivalent of 1 g. instead of 0.5 g. of pancreas was used (Figs. 1 and 2).

(Group 2) Rabbit–Synthalin A 6 mg.

Twenty-six rabbits received 6 mg. of synthalin A/kg.; 11 died and were discarded; the remaining 15 were killed between 8 and 30 hours after synthalin A injection.

Some of the alpha cells of these rabbits showed hydropic changes (Figs. 3 and 4), as described by Davis (14). The degree of alpha cell damage was graded from 0 to 3 plus. The latter grade was used when about 60% of the alpha cells were hydropic. This percentage corresponded to the maximum extent of alpha cell damage found in synthalin-A-treated rabbits. If about 30% of the alpha cells were hydropic, the case was considered a 2 plus, and 1 plus if only a few hydropic cells were present. Six animals had 0, four had 1, two had 2, and three had 3 plus lesions of their alpha cells. As seen in Fig. 1, except for rabbit S8, the hyperglycemic activity of these pancreatic extracts was comparable to that of the controls, irrespective of the degree of alpha cell lesions.

Because of the hyperglycemic activity present in the pancreatic extracts with 0, 1, 2, and 3 plus alpha cell lesions, it was considered that bio-assay of all available extracts showing less than a 3 plus lesion would be unlikely to reveal any significant information, and assays on these were not done.

(Group 3) Rabbit–Alloxan, Synthalin A 6 mg.

Eleven alloxanized rabbits received 6 mg. of synthalin A/kg. on the fourth day after alloxan injection. Twenty-four hours after this injection, the four survivors were killed.

FIG. 1. Blood sugar changes expressed in mg.% in cats injected with extracts from the pancreas of normal rabbits and of others treated with synthalin A and alloxan plus synthalin A. Dotted lines are used to facilitate the tracing out of curves. In each case extract of 0.5 g. of pancreas was assayed in a cat.

FIG. 2. Blood sugar changes expressed in mg.% in cats injected with extracts from the pancreas of dogs and rabbits treated as indicated in the figure. U—uncinate process; B—body of pancreas. Dotted lines and empty circles are used to facilitate the tracing out of curves. In each case extract of 1 g. of pancreas was assayed in a cat.

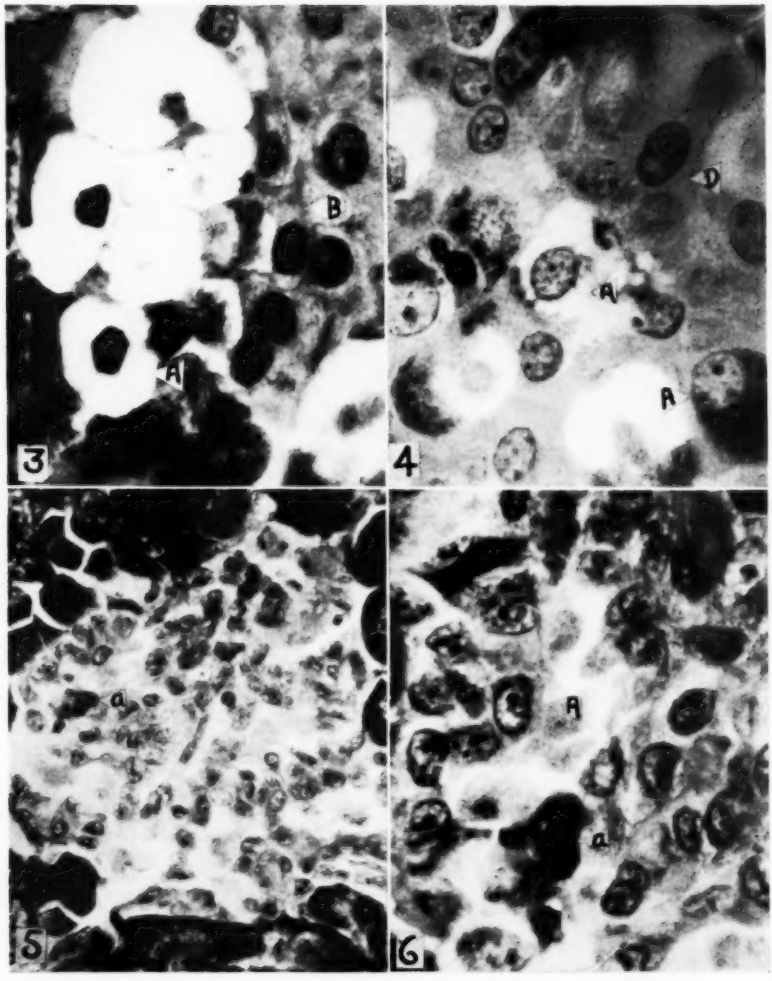


FIG. 3. Hydropic degeneration in the alpha cells of a rabbit which received 6 mg. of synthalin A., and which was killed 30 hours after. No changes are present in the beta cells. Gomori's hematoxylin. ×1300

FIG 4. Normal and hydropic alpha cells in the pancreatic islet from an alloxan diabetic rabbit which received 6 mg. of synthalin A, and which was killed 24 hours after. Alpha cells stain black. Delta cells are pale-stained, non-granular, and unaffected; beta cells are not present. Masson's trichrome. ×1300

FIG. 5. Pancreatic islet of an alloxan and cobalt-treated dog, composed mostly of alpha and delta cells. Few densely stained alpha cells (*a*) are seen. Gomori's hematoxylin. ×384

FIG. 6. Small area from Fig. 5 showing well-granulated alpha cells and some of the non-granulated, dark-stained alpha cells (*a*). ×950

FIG. 1. PANCREATIC GLUCAGON CONTENT OF RABBIT PANCREAS

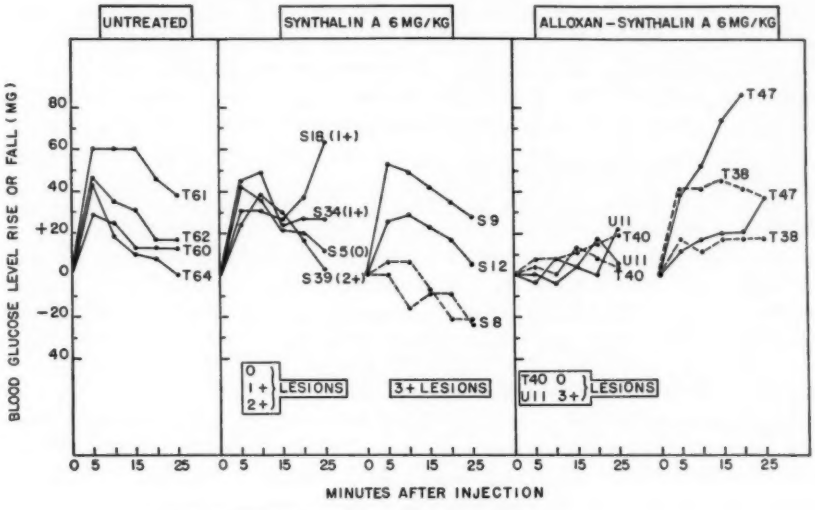
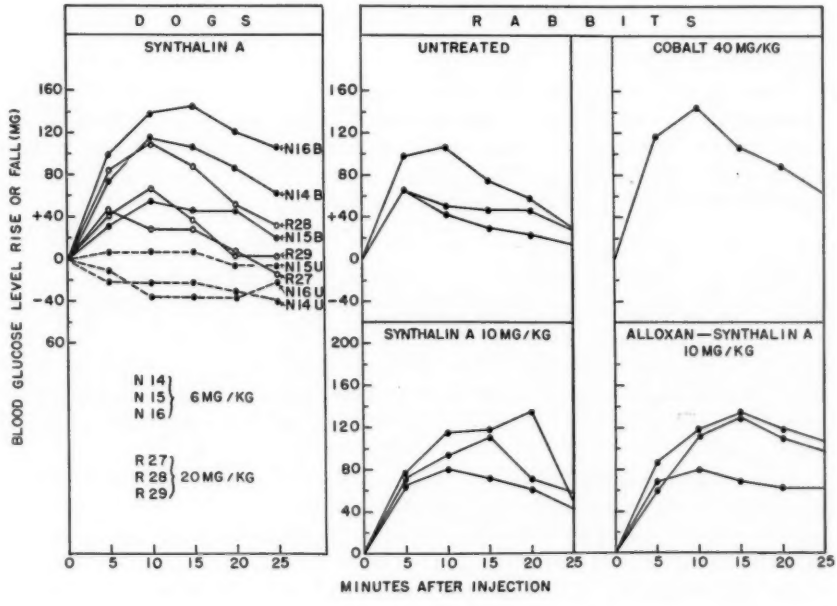


FIG. 2. PANCREATIC GLUCAGON CONTENT OF



Islet tissue was almost completely absent from the pancreas of rabbit T40, but when found, it was composed of few (5–10) normal-appearing well-granulated alpha cells. The pancreas of rabbit U11 showed numerous hydropic alpha and normal delta cells (Fig. 4); beta cells were seen only occasionally. Complete serial sections of the biopsies of rabbits T38 and T47 disclosed only fatty tissue. Moderate hyperglycemic activity was present in extracts from rabbits T40 (0 lesion) and U11 (3 plus lesions) but marked hyperglycemic activity was obtained with rabbits T38 and T47.

(*Group 4*) *Rabbit–Synthalin A 10 mg.*

Six rabbits having received 10 mg. of synthalin A/kg. according to the method of Fodden and Read (19) were autopsied immediately after death. The rabbits died between 5½ and 11 hours after synthalin A injection. Since no alpha cell lesions were noticed, their pancreatic extracts were pooled before testing. The pooled extract showed a hyperglycemic activity comparable to that of the controls (Group 1) (Fig. 2).

(*Group 5*) *Rabbit–Alloxan, Synthalin A 10 mg.*

Five alloxanized rabbits received 10 mg. of synthalin A/kg. on the fourth day after alloxan injections, and were treated thereafter as in Group 4. The rabbits died between 3½ and 10 hours after synthalin A injection. Since no alpha cell lesions were noticed, their pancreatic extracts were pooled before testing. The pooled extract showed a hyperglycemic activity comparable to that of the controls (Group 1)(Fig. 2).

(*Group 6*) *Rabbit–Cobalt*

Three rabbits were killed 24 hours after having received 40 mg. of cobalt chloride/kg. as a single intravenous injection. Pancreatic extracts from these three rabbits were pooled before testing because no change was found in the alpha cells. When tested, this pooled extract showed a hyperglycemic activity comparable to that of the controls (Group 1)(Fig. 2).

(*Group 7*) *Dogs–Synthalin A*

Three dogs, having received 6 mg. of synthalin A/kg., were killed 24 hours later. Three other dogs, having received 20 mg. of synthalin A/kg., were autopsied immediately after death, which occurred respectively at 9½, 10, and 11 hours after injection. Another three dogs, having received 25 mg. of synthalin A/kg., were all found dead 18 hours later. Glucagon extractions were not done from the last three dogs, but the pancreas was studied morphologically.

The alpha cells of none of these dogs were altered, neither was the content of glucagon of the pancreatic extracts made (Fig. 2).

(*Group 8*) *Dogs–Alloxan, Cobalt*

Six dogs received, following the method described by Carter (11), two doses of 75 mg./kg. of alloxan 24 hours apart. They were then injected

hourly for 3 hours with 300 mg. of cobalt chloride dissolved in 25 cc. of distilled water. Because the histology of the pancreas of these six dogs was similar, the extracts from the uncinate process and from the body of the pancreas were pooled from the respective areas and the pooled extracts tested separately.

No alterations were found in the alpha or delta cells nor in the glucagon content of the pancreas of these animals (Table I). Not infrequently, dark non-granular cells with the staining characteristics of an alpha cell were seen in the islet of alloxan- and cobalt-treated dogs. These cells, which may be mistaken for degenerating alpha cells following cobalt treatment were equally seen following alloxan injections, and in lesser numbers in untreated dogs (Figs. 5 and 6).

TABLE I

GROUP 8. BLOOD SUGAR RESPONSE OF FASTING ANAESTHETIZED NORMAL CATS TO INTRAVENOUS INJECTION OF PANCREATIC EXTRACTS FROM SIX ALLOXAN- AND COBALT-TREATED DOGS. (A) FROM THE BODY OF THE PANCREAS CONTAINING THE ALPHA CELLS. (B) FROM THE UNCINATE PROCESS WHICH IS DEVOID OF ALPHA CELLS

Material injected	Dose, g.	Minutes after administration of extract: Before injection	5	10	15	20	25
		Blood sugar, mg. %					
A. Pooled extract of the alpha cell-containing portion of pancreas	1	108	188	178	164	135	123
from six dogs	1	94	149	181	153	137	118
B. Pooled extract of the pancreatic tissue devoid of alpha cells (uncinate process) from the same	1	114	114	111	111	106	100
six dogs	1	100	100	97	100	118	112

(*Group 9*)

In other experiments, which will not be presented in detail, an attempt was made to confirm, by repeating their experiments as closely as possible, the work of others who claimed to have produced alpha cell damage; in this group glucagon was not assayed. A total of 18 dogs, 46 rabbits, 48 rats, and 38 guinea pigs were used. A summary of our observations follows: cobalt chloride, synthalin A, and thiodiazol-5-imid-4-aminobenzolsulphon-2-isopropyl-1-1-3,4 (I.P.T.D.) were given to rats; I.P.T.D., Orinase, and cobalt were given to dogs; I.P.T.D., Orinase, nickel chloride, and sodium-diethyl-dithiocarbamate were given to rabbits; Orinase and synthalin A were given to guinea pigs. In all these experiments on different animals only guinea pigs treated with synthalin A showed some hydropic changes in their alpha cells. This lesion was, however, very inconstant, and of much lesser intensity than when it is produced by administering cobalt.

Discussion

Hydropic degeneration of the alpha cells occurred after the administration of synthalin A in the rabbit, but not in the dog. Cobalt chloride produced no changes in the alpha cell of dogs or rabbits. This is in agreement with previous work where we showed that severe damage could be induced with cobalt in the alpha cell of guinea pigs, but not in that of rabbits (2). A more general agreement on the structural alterations, or lack of them, in the alpha cell under the influence of various drugs is necessary if progress is to be made in our knowledge of its physiological function. Very recently, a relationship of the alpha cell function with atherosclerosis has been postulated. It has been stated that alpha cells produce pancreatic elastase (11) and a hormone which regulates the cholesterol metabolism (10). Both these hypotheses were made on the basis of lesions occurring in the alpha cells of cobalt-treated dogs and rabbits, whereas, as pointed out above, no demonstrable morphological damage occurs in the alpha cells of dogs, rabbits, and rats with cobalt.

As anticipated, no reduction in the glucagon content of the pancreas of our experimental animals was observed since only minimal damage, or no damage, occurred in the alpha cells. These results are in agreement with those reported earlier where we showed that the hyperglycemic activity of pancreatic extracts was absent when the extracts were completely devoid of alpha cells (6, 7, 8) or if at least 90% of these cells were severely damaged (5). They are at variance, however, with those of Fodden and Read (19), who found no glucagon in the pancreas of synthalin-A-treated rabbits, whereas this substance persisted in the pancreas of cobalt-treated ones despite the fact that alpha cell lesions were present in both groups. The hyperglycemic activity found by these authors in the pancreas of cobalt-treated animals is better explained in the light of Fodden's more recent paper (15). In it he concluded that no lesions occur in the alpha cell of cobalt-treated rabbits. No detailed discussions were given by Fodden and Read (19) of the intensity and incidence of the alpha cell lesion of their synthalin-A-treated rabbits. It might be suggested that the difference between the results obtained by these authors and by ourselves was due to the fact that we used cats instead of rabbits for testing the extracts. In our hands, rabbits used as test animals showed false negative results when cross-checked with cats.

It seems that the lack of uniformity in the results of applying special methods of histopathological technique by different investigators, together with incomplete reporting of observations and brevity of discussion of the morphological changes of the alpha cell, has been responsible in several instances for the controversy over whether the alpha cell is the site of origin of glucagon (5, 12, 15, 19, 22, 25).

So far we have not been able to provide conclusive evidence that glucagon is produced by the alpha cell. However, the available literature, as well as the work from this laboratory are best interpreted if one considers the alpha cell of the pancreas as the site of origin of pancreatic glucagon.

References

1. BENCOSME, S. A. Am. J. Pathol. **31**, 1149 (1955).
2. BENCOSME, S. A. Rev. can. biol. **14**, 185 (1955).
3. BENCOSME, S. A. Arch. Pathol. **53**, 87 (1952).
4. BENCOSME, S. A. Am. J. Anat. **96**, 103 (1955).
5. BENCOSME, S. A. and FREI, J. Proc. Soc. Exptl. Biol. Med. **91**, 589 (1956).
6. BENCOSME, S. A., LIEPA, E., and LAZARUS, S. S. Proc. Soc. Exptl. Biol. Med. **90**, 387 (1955).
7. BENCOSME, S. A. and MARIZ, S. Can. J. Biochem. and Physiol. **34**, 779 (1956).
8. BENCOSME, S. A., MARIZ, S., and FREI, J. Endocrinology, **61**, 1 (1957).
9. BEST, C. H., HAIST, R. E., and RIDOUT, J. H. J. Physiol. **97**, 107 (1939).
10. CAREN, R. and CARBO, L. J. Clin. Endocrinol. and Metabolism, **16**, 507 (1956).
11. CARTER, A. E. Science, **123**, 669 (1956).
12. CAVALLERO, C. Rev. can. biol. **12**, 509 (1953).
13. CREUTZFELDT, W. and SCHMIDT, W. Arch. exptl. Pathol. Pharmakol. **222**, 487 (1954).
14. DAVIS. J. C. Am. J. Path. and Bact. **64**, 575 (1952).
15. DORNER, M. and STAHL, J. Compt. rend. **148**, 590 (1954).
16. FODDEN, J. H. Arch. Pathol. **61**, 65 (1956).
17. FODDEN, J. H. Am. J. Clin. Pathol. **23**, 1002 (1953).
18. FODDEN, J. H. Am. J. Clin. Pathol. **23**, 994 (1953).
19. FODDEN, J. H. and READ, W. O. Endocrinology, **54**, 303 (1954).
20. GOMORI, G. Am. J. Clin. Pathol. **20**, 665 (1950).
21. HAWK, P. B., OSER, B. L., and SUMMERSON, W. H. Practical physiological chemistry. 13th ed. Blakiston Co., Inc., Toronto. 1954.
22. KADOTA, I. and KURITA, M. Metabolism, **4**, 337 (1955).
23. STAUB, S. and BEHRENS, O. K. J. Clin. Invest. **33**, 1629 (1954).
24. VANCAMPENHOUT, E. and CORNELIS, G. Compt. rend. **145**, 933 (1951).
25. VOLK, B. W., LAZARUS, S. S., and GOLDNER, M. G. Arch. Internal Med. **93**, 87 (1954).

EFFECTS OF OVARIAN HORMONES ON THE CONTENT AND DISTRIBUTION OF CATION IN INTACT AND EXTRACTED RABBIT AND CAT UTERUS[1]

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With the technical assistance of

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Abstract

The uteri of rabbits and cats have been analyzed for sodium, potassium, and chloride, usually after various preliminary treatments with estrogen and progesterone. These tissues contain less potassium (50–80 meq./kg.) than striate muscle and more sodium (75–118 meq./kg.) than other highly cellular tissues. It appears that this cation composition can be attributed in part to a relatively large extracellular fluid volume (EFV).

Various methods have been used to estimate the EFV in these tissues. The radiosulphate space (*in vitro*) does not appear to be reliable as a measure of extracellular space. The chloride space varies, but exceeds 400 ml./kg. in all cases, and in some approaches 700 ml./kg. Inulin space (*in vitro*) is about 60% of the chloride space, which in turn is usually smaller than the sodium space. The chloride space appears to provide the best approximation to the EFV since its volume of distribution rarely exceeds the sodium space, and since chloride (but not sodium) can be removed completely on leaching in isotonic sucrose.

Calculated cellular potassium concentrations are as high as or higher (150–210 meq./l.) than in striate muscle. Apparently the low total tissue potassium concentration is a consequence of the large EFV.

Appreciable quantities of sodium (20–50 meq./l.) reside outside of the chloride space in most cases, presumably in cellular water. Furthermore, a residue of sodium remains in uterine tissue after leaching in isotonic sucrose or choline chloride. With appropriate leaching procedures, an initial rapid depletion of tissue sodium is followed by a period of relatively slow loss, indicat ng derivation of sodium from at least two separate tissue spaces. Equilibration in isotonic potassium chloride causes nearly complete equilibration of potassium and chloride throughout tissue water, but does not remove residual sodium, suggesting chemical binding rather than Donnan distribution as the mechanism of sodium retention.

The effects of estrogen and progesterone on the concentrations of cations in uterine cells are shown to be relatively small. Estrogen causes expansion of the cellular compartment relative to the extracellular space in both rabbit and cat and decreases the concentration of cation (per liter of tissue water and per liter of intracellular fluid). Progesterone treatment, given after estrogen, interfered with the ready entrance of chloride into the cellular space of rabbit uterus exposed to isotonic choline chloride. Cat uterus was not so affected, there being very little penetration of chloride even after estrogen alone.

Introduction

As yet the mechanism of action of ovarian hormones on the functions of the uterus has not been elucidated, although studies in various systems have demonstrated numerous changes initiated by these steroids (14,18). The cell membrane, and its activity in selectively accumulating and extruding ions, may be the site of important and perhaps crucial hormonal effects. However, before effects of hormones on cellular concentrations of electrolytes

[1] Manuscript received in original form February 25, 1957, and, as revised, September 6, 1957. Contribution from Department of Pharmacology, Faculty of Medicine, University of British Columbia, Vancouver, Canada.

can be studied effectively, methods for differentiating cellular and extracellular changes must be sought. The characteristics of electrolyte distribution in uterine tissues also must be clarified.

Previous studies of rat and Macaque uteri (21,22) have indicated that estrogen causes an increase in tissue water concentration. In the rat, initial imbibition of water without cellular growth was followed by growth of tissue with increased water concentration. Studies of the electrolytes in rat uterus indicated that estrogen, during the premitotic phase of its action, increased the sodium concentration of the tissues and decreased the potassium concentration. This increase in sodium concentration was not accompanied by an increase in "chloride space" sufficient to contain the increased sodium. Thus estrogen appeared to have caused a movement of sodium into cells (see also 13). However, since total chloride space always exceeded the sodium space, it appeared that chloride too was present in uterine cells. Since the validity of various measures of extracellular volume is still uncertain, the localization of estrogen-induced changes of fluid and electrolyte in cellular and extracellular compartments remains in doubt.

The effects of estrogen on uterine electrolytes have not been studied previously in the rabbit. While the influence of progesterone on uterine electrolyte composition has been investigated in the rabbit (12), these data did not provide a direct estimate of the chloride spaces.

In this report the effects of both an estrogen and progesterone on uterine electrolytes in the rabbit have been studied. Several measures of the extracellular space have been compared and evaluated. Other means of determining ion distribution also have been examined. In addition, because of the contrasting effects of ovarian hormones on the contractile responses of the cat uterus (19), similar data for this species have been collected and compared with the results obtained in rabbits. The results indicate that these tissues may have unique patterns of control of electrolyte distribution.

Methods

Source of Preparations

The uteri of immature (6–7 weeks old) albino rabbits and immature (<1.0 kg.) cats were studied, both with and without hormonal pretreatment.

Hormone Pretreatment

(1) *Estrogen Treatment*

Immature rabbits and cats were given 100 μg. per day of diethylstilbestrol dipropionate subcutaneously in oil for 6 days prior to killing (the dose of this estrogen was cut in half for kittens weighing 0.5 kg. or less). In rabbits, this procedure caused uterine growth from an initial 0.3–0.5 g. to 1.4–5.9 g., and the development of characteristic responses to oxytocic agents. Kittens appeared to be more susceptible to the toxic effects of the regime (diarrhea, death, etc.) and the increase in uterine weight achieved was quantitatively much less than in the rabbit (from an initial 0.4–0.5 g. to 0.5–1.5 g.).

(2) *Estrogen and Progesterone Pretreatment*

One group each of rabbits and cats was continued on estrogen for 3 additional days while progesterone dipropionate[2] in oil was added (2 mg. per day given subcutaneously at a separate injection site). (In a few cases rabbits were treated with estrogen alone for 9 days, but uteri from these animals showed no significant differences from those treated for 6 days and are included with the others.)

Collection and Preparation of Samples

Rabbits were killed by a blow at the foramen magnum, and the uterus rapidly excised, free from mesentery and large blood vessels, and from the periuterine fat. After adhering blood had been removed by gentle blotting, the uterus or a portion of it was placed in a tared beaker for weighing.

Cat uteri were removed under barbiturate anaesthesia (30 mg./kg. pentobarbital sodium given intraperitoneally).

Each uterus was subdivided into several pieces. The horn segments just adjacent to the Fallopian tubes were used for these studies since control studies indicated the occurrence of variation in electrolyte contents between tubal and cervical portions. The other pieces were used for *in vitro* experiments on electrolyte exchange which are to be reported elsewhere.

In some animals from both species a sample of heart blood for electrolyte analysis was taken into heparinized syringes about two minutes prior to removal of the uterus. The samples were discarded if detectable hemolysis occurred.

Analytical Procedures

All tissue pieces were weighed wet, dried for 5 or 6 days at 105° C., and then reweighed. Fat content was negligible. The samples analyzed were too small to permit haemoglobin determinations as well as complete electrolyte analyses, but blood content did not appear to be an important variable, since procedures designed to vary blood content did not markedly alter electrolyte concentrations.

Tissues used for chloride analysis were dried and pulverized thoroughly. When possible, one aliquot of the dried tissue was taken for chloride analysis and the remainder set aside for cation determinations. Tissue chlorides were determined by the method of Van Slyke as modified by Wilson and Ball (23, 26).[3] All chloride titrations were carried out at 4° C. Plasma chlorides were determined by the method of Schales and Schales[3] (20,1).

Sodium and potassium were determined with the Janke flame spectrophotometer using lithium as an internal standard. For tissue cation analysis,

[2] The progesterone dipropionate (Lipolutin) 50 mg./ml. was kindly donated by Parke, Davis Company.

[3] The accuracy of the Van Slyke method for tissue chloride (liver and whole carcass) has been questioned recently by Williams *et al.* (25) and by Cheek and West (4). Careful studies in this laboratory have indicated that this method is accurate provided only that as much as two microequivalents of chloride are present. Further, in uterine tissue, prior KOH digestion did not increase the analytical values. Therefore it seems likely that the values reported are approximately correct. In pieces leached in sucrose, with very low chloride content, the limitations mentioned above increased the likelihood of error.

the whole dried tissue or an aliquot of the dried ground tissue was digested in concentrated nitric acid. After digestion, the white crystalline residue was redissolved in demineralized distilled water with a few drops of 1 *N* hydrochloric acid and diluted to a convenient volume based on dry weight. Sodium and potassium in plasma or solutions were determined without digestion at a dilution of 1 : 200. At rapid atomizer flow rates, the presence of sucrose had no effect on the analyses of sodium and potassium. Since it was not always possible to examine chloride as well as sodium and potassium in the same piece, the intracellular cation contents were calculated from the averages of groups rather than from individual pieces.

The possibility that gain of sodium from the glassware used in digestion or dilution might produce a significant error when the tissue sodium content was very small (residue after leaching) has been considered. Pyrex glassware was used throughout, and the apparent sodium concentration in nitric acid blanks subjected to the same procedures as experimental samples was negligible. Sodium determinations were carried out immediately after dilution of the samples, since the sodium concentration did increase slightly on prolonged standing in pyrex containers.

Inulin and Radiosulphate Space

Inulin was determined by the anthrone procedure of Young and Raisz (27). The inulin space of uterine segments was estimated *in vitro* by equilibration of the tissue pieces for 3 hours in 5 ml. of Krebs–Ringer solution containing a measured concentration of inulin (about 1500 μg./ml.) and subsequent determination of the inulin uptakes by leaching for another 3 hours into 5 ml. of an inulin-free Krebs–Ringer solution. The inulin concentration in the leaching medium was then determined.[4] The following precautions were necessary in order to obtain reproducible results: (1) Viability was assured by maintenance of temperature at 37° C., by continuous bubbling of carbogen (95% O_2: 5% CO_2) through the medium, and by provision of an adequate supply of glucose as a source of energy. (2) Uterine segments of less than 500 mg. mass were used to ensure complete equilibration within the interval employed. (3) After initial equilibration, the uterine segment was very rapidly rinsed twice in inulin-free Krebs–Ringer before transfer to the medium used for leaching in order to remove the inulin-containing solution adhering to its surface.

Recently the radiosulphate volume of distribution also has been advocated for extracellular space estimation (24). Studies of the *in vitro* radiosulphate space were carried out using the same principle as with inulin.[5] Following an initial phase of equilibration (30–40 minutes), the radiosulphate space continues

[4] The general formula used for determining the volume of distribution of a substance *in vitro* was:

$(C_F \times \text{vol.}) / (C_I - C_F) \times 1/\text{tissue weight (kg.)} = \text{space (ml./kg.)}$;

C_I = concentrations in initial bath medium (μg./l.).

C_F = concentrations in final bath medium (μg./l.).

Vol. = volume of bathing medium added for final equilibration.

[5] Sulphur^{35} was counted in a flow counter after 1 ml. of the bathing medium was dried. Good geometry was obtained by coating the planchets with a film of saureisen.

to expand at a fairly rapid rate so that by 120 minutes it reaches 600–700 ml. per kilogram. Since extensive cellular penetration or combination is indicated, the radiosulphate space is not a reliable or accurate measure of the extracellular space of tissues *in vitro*.

Ion Extraction Procedures and Solutions Employed

In some cases uterine cation distribution was studied by leaching into an electrolyte-free solution (sucrose $M/3$) or into a sodium-free solution containing chloride at 162.5 meq./liter (choline chloride or potassium chloride) or into a bicarbonate–Ringer containing sodium and chloride but no potassium (Na, 156.5; Cl, 136; HCO_3, 20.5 meq./l.). After the tissue had been weighed, the usual precautions were taken to ensure tissue viability, although substrate was lacking in sodium-free solutions. Tissues were incubated in oxygenated Krebs–Ringer solution at 37° for a recovery period of 20 minutes before transfer into the leaching medium.[6] After two rapid rinses in the leaching fluid, tissues were extracted for a period of 1 to 8 hours. Tissue and medium were then analyzed for sodium, potassium, and chloride. In another series of experiments, following the 20 minutes recovery period, the medium was replaced every 7 minutes (the approximate time required for equilibration of medium with extracellular ions), so as to maintain a maximal concentration gradient into the medium. All samples of media were collected and analyzed to provide a continuous indication of the rate of ion leaching.

During leaching, changes in the concentrations of tissue water and total solids varied according to the composition of the extraction medium. When the tissue extracellular fluid was replaced with isotonic sucrose, the large molecular weight of this substance led to an increase in total tissue solids, while the opposite effect occurred when extracellular electrolytes were replaced with choline chloride. However, in both of the media, the tissues lost water and hence their total weight decreased. On the other hand, when tissues were leached in saline–bicarbonate, or potassium chloride, an increase in tissue weight and water concentration was observed, apparently the result of cellular uptake of the water and electrolyte from these media. These changes were marked in rabbit uterus, but much less, or absent, in cat uterus. To permit comparison of residual tissue electrolytes after leaching in these various media, the data have been calculated in terms of the initial fresh weight of the tissues.

Results

(A) Effects of Estrogen and Progesterone on Tissue Cation Concentrations

(1) *Rabbit Uterus*

The sodium and potassium concentrations in uterine horn tissue taken from hormone-treated immature rabbits and from control animals are shown

[6] The recovery period of 20 minutes in Krebs–Ringer solution usually resulted in a slight increase in the chloride space, and a somewhat larger increase in the sodium space, but no detectable change in tissue potassium concentration occurred.

TABLE I

CONCENTRATION OF SODIUM AND POTASSIUM IN IMMATURE RABBIT UTERINE HORNS, TUBAL PORTION*

Treatment	No. animals	No. pieces	Uterine wet wt., g.	% H_2O	meq./kg. wet		meq./kg. dry	
					K	Na	K	Na
Control	5	6	0.39 (±0.15)	79.2 (±1.2)	53.9 (±1.9)	89.9 (±2.6)	266 (±30.5)	451 (±24.5)
+Estrogen	34	52	2.8 (±0.16)	84.0 (±0.4	67.4 (±0.8)	79.0 (±0.6)	437 (±12)	515 (±13)
+Estrogen and progesterone	14	17	3.89 (±0.33)	81.6 (±0.6)	68.3 (±1.3)	75.0 (±1.1)	376 (±10.5)	413 (±10.0)

* Variability expressed as standard error in this and subsequent tables.

TABLE II

CONCENTRATIONS OF SODIUM AND POTASSIUM IN IMMATURE CAT UTERINE HORNS, TUBAL PORTION

Treatment	No. animals	No. pieces	Uterine wet wt., g.	% H_2O	meq./kg. wet		meq./kg. dry	
					K	Na	K	Na
Immature	3	4	0.44 (±0.04)	81.9 (±1.5)	51.1 (±2.8)	118.0 (±13.2)	274 (±3)	632 (±43)
+Estrogen	12	13	0.98 (±0.07)	83.5 (±0.2)	62.4 (±1.6)	91.7 (±2.7)	391 (±14)	568 (±20)
+Estrogen and progesterone	5	7	1.43 (±0.22)	82.0 (±0.3)	67.1 (±2.2)	76.7 (±3.7)	370 (±12)	422 (±22)

in Table I. An exceptionally high tissue sodium concentration is evident in all uterine samples. Simple estrogen treatment decreased the ratio of sodium to potassium either by increasing the potassium concentration or by decreasing the sodium concentration or both. Average water concentration also was increased by estrogen. Alteration of the estrogen-induced pattern of electrolyte distribution as a result of additional treatment with progesterone usually consisted of a slight reduction in sodium and water concentration, with little effect on potassium (Table I).[7]

(2) *Cat Uterus*

The sodium and potassium concentration of immature and hormone-treated cat uterine horns are presented in Table II. These data indicate that treatment with ovarian hormones produced qualitatively the same effects in the cat as in the rabbit. The slight increase in potassium concentration following progesterone treatment was not significant, but there was a definite decrease in the sodium–potassium ratio.

(B) Distribution of Uterine Electrolytes

(1) *Based on Estimates of the Extracellular Fluid Volume (EFV) of Uterine Tissues*

In order to localize alterations in tissue water and electrolytes, it is necessary to estimate the volume and electrolyte concentration of extracellular fluid. It is clear that the plasma electrolyte concentrations (and presumably extracellular fluid electrolyte concentrations) are not significantly altered by the various hormonal treatments (Table III).

There is no completely satisfactory method for measuring the EFV. Errors are derived either from the penetration of cells by the various substances used (as with sodium and to a lesser extent with chloride) or from failure to obtain equilibrium with the whole extracellular space, especially acellular connective tissue (as with inulin (17)). Therefore the inulin space represents a minimal and chloride space a maximal estimate of EFV. The EFV of uterine tissue is relatively large (which may account, in part, for the high sodium and low potassium concentration in this organ).

Inulin space determinations obtained for uterine segments *in vitro* showed satisfactory reproducibility (Table IV). These are compared with values for chloride and sodium spaces, calculated as described by Manery (15).

[7] Occasionally, in some rabbit uteri which were colored dark cherry red (apparently due to congestion or engorgement with venous blood) progesterone appeared to produce a different effect on electrolyte distribution. In these uteri the water and sodium concentration (and chloride space) were significantly higher than similarly treated pieces of lower water concentration. This difference was not due to a systematic error, since all cervical pieces were found to be members of the same population with respect to water concentration (all greater than 84%), even though these pieces were handled in the same manner as those from the horns. Likewise, no groupings on the basis of water concentration were found in the analyses of cat uteri or estrogen-treated rabbit uteri. Calculations show that differences could not be explained solely by either an increase in blood content or by an increase in extracellular fluid. Both changes probably occurred. Wide-spread hypermia and edema have been reported by Gillman (10) to occur following treatment with certain combinations of estrogen and progesterone. These tissues have been excluded from Table I.

TABLE III

PLASMA CONCENTRATIONS OF ELECTROLYTES

Species	Condition	Meq./l. plasma		
		Na	K	Cl
Rabbit	Immature, no treatment	145.9 (3) ±1.7	4.20 (3) ±0.50	99.6 (3) ±1.43
	Estrogen	145.2 (7) ±1.3	3.92 (7) ±0.15	103.4 (7) ±1.84
	Estrogen and progesterone	143.8 (8) ±1.3	3.95 (8) ±0.14	105.4 (8) ±1.56
Cat	Immature, no treatment	——*	——	——
	Estrogen	159.0 (5) ±2.4	3.74 (5) ±0.17	121.4 (5) ±1.75
	Estrogen and progesterone	153.5 (6) ±1.0	3.51 (6) ±0.17	123.6 (6) ±1.90

NOTE: Use 930 ml./kg. for plasma water content in calculations of extracellular ion concentrations.

* In cases where sufficient data not available, use average for species in calculations.

While a slight increase in chloride space was observed in tissues incubated in Krebs solution, inulin spaces *in vitro* are considerably lower than the chloride space of control tissues. The average ratio of the inulin space to the chloride space is about the same in the uterus (0.60) as that reported for the whole body (0.63) by Gamble and Robertson (9), although, in uterine tissues, this value varies considerably with age and hormonal status.

The data in Table IV demonstrate that a great deal of the sodium and probably some of the chloride of uterine tissue was located within the cells or in connective tissue not readily penetrated by inulin. Obviously, too, the sodium space exceeded the chloride space in almost every case.

Hormone-Induced Alterations

These data also indicate that estrogens decreased the EFV (chloride space) of both cat and rabbit uteri, although the variation from one uterus to another was great. Progesterone had no further marked effect on estimated EFV (chloride space) in either species. In general, changes in the inulin and sodium spaces paralleled alterations in the chloride space, but this was not invariably so. The sodium space of immature cat uteri was relatively large while that of progesterone- and estrogen-treated specimens approximated the chloride space.

Calculations of cellular cation concentrations have been made (Table V) using these estimates of extracellular space and the data previously summarized. The calculated cellular potassium concentration was at least 100 meq./l. of cell water in all instances. In addition the total cation concentration was uniformly greater than that of the extracellular fluid (at least 184 meq./l. cell water), although the sodium concentration in cells varied greatly.

TABLE IV

TISSUE SPACES OF RABBIT AND CAT UTERINE HORN (IN CC./KG.)

Species	Condition	Inulin space *in vitro*	Chloride space	Sodium space*	Radio-sulphate space *in vitro*	Ratio inulin sp.: chloride sp.
Rabbit	Immature	270 ± 30 (9)	573 ± 35.9 (7)	613		0.471
	+Estrogen	240 ± 20 (12)	468 ± 15.1 (14)	551	554	0.513
	+Estrogen and progesterone	240 (2)	438 ± 17.7 (6)	509	427	0.548
Cat	Immature	405 ± 20 (6)	558 ± 14.9 (4)	751	—	0.726
	+Estrogen	390 ± 30 (6)	482 ± 42.8 (6)	571	631	0.809
	+Estrogen and progesterone	310 (2)	505 ± 29.7 (5)	495	455	0.614

NOTE: Numbers in parentheses indicate number of pieces analyzed.
* Sodium space from data in Tables I, II, and III using 0.94 as Donnan Factor.

TABLE V

CONCENTRATIONS OF SODIUM AND POTASSIUM IN INTRACELLULAR WATER OF RABBIT AND CAT UTERI (CALCULATED ON BASIS OF DIFFERENT TISSUE SPACES)

		Inulin space, meq./kg.			Chloride space, meq./kg.		
Species	Condition	K	Na	Total	K	Na	Total
Rabbit uteri	Immature	100.7	101.3	202.0	236.4	29.4	265.8
	Estrogen	108.9	77.3	186.2	170.9	30.8	201.7
	Estrogen and progesterone	117.1	74.5	191.6	176.3	31.8	208.1
Cat uteri	Immature	120.0	143.5	263.5	213.8	119.6	333.4
	Estrogen	137.1	74.2	211.3	153.2	52.0	205.8
	Estrogen and progesterone	129.7	63.5	193.2	207.9	−2.6	205.3

Estrogen decreased the apparent potassium concentration in uterine cells of both species when chloride space was used as an estimate of the EFV (but not when the inulin space was used). Estrogen also decreased the calculated sodium concentration of uterine cells in cat uteri regardless of whether chloride or inulin space was used to estimate EFV. However, in the rabbit, this effect was less certain, being apparent only when the inulin space was used. On one point however, all data agreed: the total cellular cation concentration (sodium plus potassium) was decreased by estrogen.

Progesterone had very little effect on cellular cation concentrations in the rabbit uterus, irrespective of how EFV was estimated. In cat uteri, however, progesterone caused a decrease in cell sodium, especially when chloride space was used as the EFV.

(2) *Based on Loss of Tissue Sodium and Chloride into Isotonic Sucrose and Choline Chloride*

The localization of the described hormone-induced alterations in fluid and electrolyte remains uncertain although the actual concentrations of electrolytes in cells must have been within the limits set by calculations from

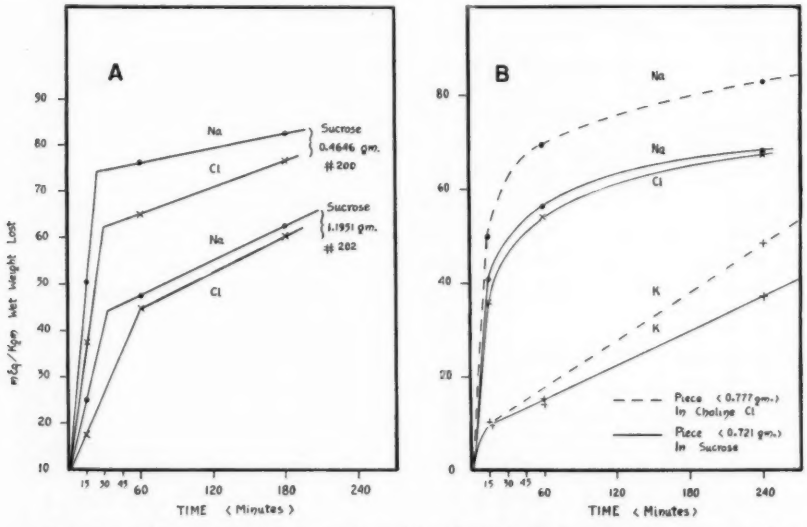


FIG. 1. A. The effect of variation in weight of rabbit uterine pieces (pretreated with estrogen and progesterone) on the rate of loss of sodium and chloride into isotonic sucrose *in vitro*. The volumes of distribution for the rapidly equilibrating fraction of sodium and chloride can be calculated by extrapolation of the slower rate back to zero time and division of the ion content so obtained by the concentration of that ion in Krebs–Ringer solution. These volumes, for sodium and chloride respectively, were: in the larger pieces; 288 and 291 ml./kg. tissue, and in the smaller piece; 526 and 464 ml./kg.

B. The effect of the composition of the leaching solution on the rate of loss of rabbit uterine electrolytes (estrogen pretreatment). In choline chloride, both sodium and potassium were lost at a faster rate than in sucrose. The volume of distribution of the rapidly emerging sodium was 461/ml./kg. in choline chloride and 378 ml./kg. in sucrose.

the inulin and chloride spaces. Presumably the cell membrane constitutes a mechanical or electrochemical barrier to the outward diffusion of some cellular ions particularly cations. It seemed feasible to try to localize at least some ions by following their diffusion from uterine tissue *in vitro*. Uterine electrolytes were allowed to equilibrate for an hour or more with isotonic sucrose, and the concentrations of ions determined at various intervals in the medium and finally in the tissues. Though the estimated spaces (per unit weight of tissue) required to contain the rapidly diffusing portions of sodium (or chloride) were often nearly identical, this volume was always larger than the inulin space, and was influenced by several variables. These included variation in mass of tissue, substitution of choline chloride for sucrose as the leaching fluid, and variation in the interval at which the electrolyte content of the leaching medium was determined. Fig. 1 illustrates the effect of variation in mass and composition of the leaching fluid on cation extraction.

While the rates of diffusion of sodium (or chloride) from the extracellular space were not sufficiently rapid and uniform to permit an accurate estimation of the quantity of these ions outside cells, nevertheless it is apparent that the loss of sodium and chloride followed a pattern different from that of potassium. An early rapid loss of chloride and sodium was followed by a continually decreasing rate of loss, while potassium extraction proceeded at a uniform rate, after the initial period of rapid loss.

Repeated replacement of the leaching medium prevented the development of equilibrium and permitted a more detailed analysis of the rates at which sodium and chloride were being lost from the tissue (Fig. 2). The analysis of the curves of sodium emergence show clearly that sodium also emerges from at least two distinct tissue sites in these experiments. Thus sodium undergoes an initial high but very rapidly decreasing rate of loss until the 35 minute sample. From 35 to 63 minutes, successive samples received relatively constant amounts of sodium. Increments of sodium lost to the medium during this latter phase were very small and therefore subject to to more error in determination. The chloride extrusion curve in sucrose followed the same initial pattern as the curve for sodium. However, in subsequent samples, the bathing medium became virtually chloride-free, indicating that the tissues had lost most of their freely diffusible chloride. In the initial samples, the average ratio of sodium to chloride extruded, was approximately the same as that in the Krebs–Ringer with which the tissues had previously equilibrated. This ratio increased markedly toward the end of the experiment, indicating that terminally sodium was being derived from a space containing little free chloride. Chloride output approximated the total chloride content of the control tissue, if precautions were taken to avoid inclusion of extra chloride in the fluid adhering to the tissue surface upon removal from the recovery medium. This was accomplished, in some experiments, by rapid rinsing of the tissue in the chloride-free solution before transfer to the leaching medium. Even after such rinsing, total sodium

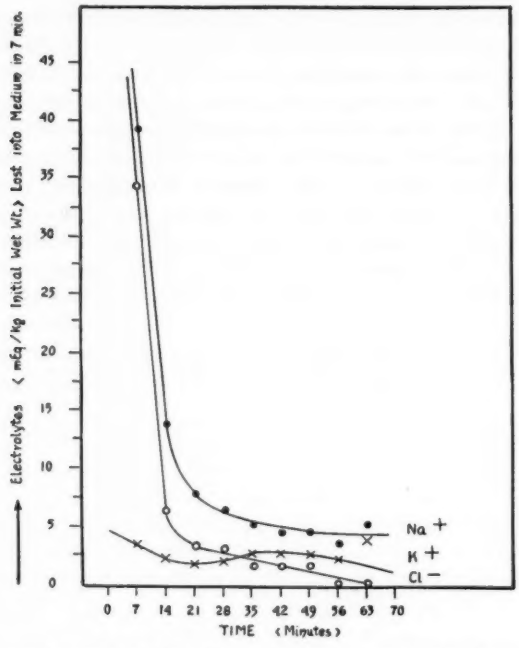


FIG. 2. Extraction rate of Na, K, and Cl from a cat uterine horn (estrogen plus progesterone pretreatment) into $M/3$ sucrose with successive replacement of the medium every 7 minutes. See text for details.

recovery (extracted plus residue) exceeded the sodium content of control pieces, as a result of the uptake of sodium and water in excess of chloride during incubation in the recovery medium.

These data suggest that the diffusibility of an appreciable fraction of the tissue sodium is limited, whereas all of the chloride seems freely diffusible from these tissues. Residual tissue electrolytes in both types of experiments were determined (Table VI). Data on residual electrolytes, following exposure to a medium containing sodium (saline–bicarbonate) are included for comparison. These data clearly confirm the presence of a non-diffusible sodium fraction. The residual sodium content (10–20 meq./kg. original wet weight or 20–40 meq./liter of cellular water) was equivalent to roughly two-thirds of the initial cellular sodium content calculated using chloride space as an estimate of the EFV. The concentration of residual tissue sodium was significantly greater than that in the final leaching medium (less than 0.2 meq./l. in experiments with exchange of medium, and 4–7 meq./l. in experiments in which sodium was allowed to accumulate in the medium), whereas there was little residual chloride after leaching into sucrose (final concentration in medium from 0–6 meq./l.). Prevention of accumulation of sodium in the external medium increased the amount of sodium extracted

TABLE VI

RESIDUAL SODIUM AND CHLORIDE AFTER LEACHING OF UTERINE SEGMENTS IN ARTIFICIAL MEDIA, MEQ./KG. ORIGINAL WEIGHT

Species	Hormonal status	Equilibration without replacement of media								Successive replacement sucrose/Na
		Sucrose		Choline chloride		Cl space	Saline bicarb.		Cl space	
		Na	Cl	Na	Cl		Na	Cl		
Rabbit	Estrogen	12.5 (8)* ±1.0	3.2 ±0.9	7.6 (8) ±0.9	113.6 ±1.6	698	105.5 (30) ±2.7	75.9 ±1.6	560	15.5 (6) ±2.2
	Estrogen and progesterone	13.5 (8) ±1.5	0.6 ±0.1	11.8 (8) ±0.5	79.4 ±3.5	488	104.7 (30) ±2.4	70.5 ±1.4	520	19.4 (6) 0.8
Cat	Estrogen	17.6 (8) ±1.9	4.8 ±2.8	13.7 (10) ±1.9	89.3 ±5.2	549	—	—		26.1 (4) ±2.7
	Estrogen and progesterone	13.1 (7) ±1.0	5.6 ±3.4	17.5 (9) ±2.8	87.7 ±3.9	539				20.5 (2)
Duration of leaching, min.		240		240			70			63–70

* Value in parentheses = number of pieces studied.
NOTE: Tissue volume accessible to chloride diffusing in from the medium expressed in ml./kg. original weight.

and decreased residual tissue sodium when similar durations of exposure were compared. Sodium extraction was more rapid and apparently more complete when choline chloride replaced sucrose as the leaching medium for rabbit uteri, as expected from the determinations of the rate of ion accumulation in the medium (Fig. 1B). The non-diffusible fraction of tissue sodium could be explained either by distribution in cells according to the Gibbs-Donnan principle, or by "binding" (chemical combination) with some tissue constituent. However, when pieces of uterine tissue were leached in isotonic potassium chloride, the residual sodium content was as great as that found in other extraction media (Table VII (B)). The equilibration of potassium and chloride between tissue and medium was as complete (Table VII (B)) as could be expected in view of the presence of fixed tissue anions.

TABLE VII

EFFECT OF 4 HOURS' LEACHING IN ISOTONIC KCl ON RESIDUAL UTERINE ELECTROLYTES (162.5 meq./l. K and Cl)

		(A) meq./kg. original wet wt.			(B) meq./l. tissue H_2O		
		K	Na	Cl	K	Na*	Cl
Rabbit	E (5)	206	12.4	182	167	10.0	147
	E + P (4)	198.5	12.5	163	171	11.0	140
Cat	E (2)	128.0	17.7	109	142	19.7	133

* Final Na concentration in medium from 2 to 6.0 meq./liter.

Hormone-Induced Alterations

Hormonal pretreatment had relatively minor effects on the residual tissue sodium content in the various extraction media (Table VI) though progesterone increased it in rabbit uterus. Estrogen-treated rabbit uteri revealed a remarkable enlargement in the tissue volume accessible to chloride diffusing in from the external medium, particularly when sodium and potassium were absent from the leaching fluid (choline chloride medium). This property was not possessed by estrogen-treated cat uteri, and, in rabbits, was effectively eliminated (cf. Table IV) in uterine pieces from animals treated with progesterone as well as estrogen.

Effect of Temperature on Residual Electrolyte Content

The effect of temperature on the rate and degree of ion extraction was also of interest in relation to the location of tissue electrolytes. Decrease in temperature clearly slowed extraction of tissue potassium into sucrose much more than that of chloride or sodium, indicating that the mechanisms controlling the tissue distribution of extractable sodium and chloride were relatively temperature insensitive, while those controlling potassium were highly sensitive to temperature changes (Table VIII (A)). Analyses of residual tissue electrolytes confirmed these findings. Neither the final sodium content,

TABLE VIII

EFFECT OF TEMPERATURE ON EXTRACTION OF UTERINE ELECTROLYTES IN SUCROSE

(A) Ions (meq./kg. original weight) extracted into medium

	Na		Cl		K	
T°	120 min.	325 min.	120 min.	325 min.	120 min.	325 min.
Rabbit 206-E						
4°	82	93	73	82	8	13
24°	93	100	75	86	9	19
37°	88	95	89	92	23	43

(B) Ions (meq./kg. original weight) remaining in tissue after leaching 6 hours

	Na		Cl		K	
	37°	4°	37°	4°	37°	4°
Choline Cl						
Cat 124-E	13.0	14.3	104.8	83.1	16.4	46.8
Rabbit 205-E	9.7	15.4	111.1	113.6	7.9	52.7
Sucrose						
Cat 126-E	20.2	18.6	5.2	1.3	7.8	40.3
Rabbit 206-E	11.8	11.1	0.9	3.5	14.4	38.7

(C) Ratio meq./l. final tissue H_2O: meq./l. bath solution

	Na		Cl		K	
	37°	4°	37°	4°	37°	4°
Choline Cl						
Cat 124-E	2.4	3.2	0.86	0.64	5.9	55.5
Rabbit 205-E	3.4	6.1	0.84	0.88	3.9	103.8
Sucrose						
Cat 126-E	6.1	5.9	1.06*	0.39*	9.3	44.6
Rabbit 206-E	3.9	3.5	0.33*	1.30*	8.4	75.5

* Subject to large errors owing to low concentration in meq./l. tissue H_2O.

nor the final ratio of tissue to extracellular concentration of the ion was affected significantly by reduction of temperature to 4° C. in either leaching medium (Table VIII (B,C)). In contrast, the ratio of the potassium concentration of the tissue to that in the medium was increased from less than 10 : 1 to about 50 : 1 or more by lowering the temperature to 4° C. The very small amounts of residual chloride after leaching in sucrose indicated nearly complete extraction of this ion at both temperatures.

Discussion

The data reported in the present study do not permit a distinction between the contribution of the endometrium and the myometrium to the over-all electrolyte composition of uterine tissue. Progesterone has been reported to alter the ionic composition of these two tissue components in opposite

directions (12). However, more recent analyses of isolated strips of uterine longitudinal muscle (8*a*) have indicated that the electrolyte composition of myometrium does not differ qualitatively from that of the entire uterus.

1. Chloride Distribution in Uterine Tissue

While the chloride space represents an upper limit for the estimates of the EFV in uterine tissues, it is not equivalent to the true EFV in some instances. In all uteri studied, tissue chloride is almost completely diffusible upon extraction into chloride-free media, and in most instances, the calculated chloride space does not exceed the sodium space. Further, the chloride space of cat uteri and progesterone-treated rabbit uteri *in vitro* is relatively constant in solutions of varied chloride composition. However, the marked expansion of the chloride space in chloride-containing media *in vitro* on the part of estrogen-treated rabbit uteri is unique. It is difficult to account for this enhanced chloride uptake in terms of enlargement of the extracellular space, and presumably increased cellular penetration of the ion is involved. This finding demonstrates that the permeability of uterine cells to chloride *in vitro* is influenced by naturally-occurring substances (hormones) and makes it difficult to exclude some cellular penetration of the ion *in vivo*. In fact, the chloride space does exceed the sodium space in certain circumstances (in some cat uteri).

The retention of significant quantities of residual chloride in estrogen-treated rabbit uteri after equilibration with sucrose also supports the hypothesis that some chloride is intracellular in these tissues. A similar situation appears to hold in the case of rat uteri (21).

The studies cited have not permitted a distinction between the endometrial and myometrial tissue components. However, analyses of human myometrium (8) and intestinal and uterine longitudinal muscle (8*a*) have indicated that chloride in substantial quantities is present in some smooth muscle cells. The occurrence of chloride in smooth muscle cells may be a widespread phenomenon. The factors controlling the penetration of myometrial cells by chloride, and the relation of altered chloride permeability to uterine activity warrant further study.

2. Sodium Distribution in Uterine Tissue

Rabbit and cat uteri have a higher sodium concentration than has been reported for other tissues, except acellular connective tissue (15). Somewhat lower values for the sodium concentration of uterine tissue have been reported by Horvath (12). His data are based chiefly on the measurement of cations extracted into solutions of lithium salts. Our finding of substantial quantities of residual tissue sodium after many hours of leaching casts doubt on the validity of values for tissue sodium which depend upon extracted ion. Our data indicate that a considerable portion of uterine sodium was either in cells or in connective tissues as judged by comparison of the inulin, chloride, and sodium space measurements. A non-extracellular site for a considerable portion of the uterine sodium also was suggested by the finding that substantial

quantities of residual sodium were found in the tissue after *in vitro* equilibration with isotonic sucrose, choline chloride, or potassium chloride, whereas tissue chloride was exhausted by leaching in sucrose. Meigs (16) reported that frog stomach muscle lost only 40% of its sodium on exposure to isotonic sucrose for several hours, and suggested that residual sodium is located partially or wholly in the muscular portion of this tissue. Harris and Steinbach (11) have recently suggested that residual sodium in frog skeletal muscle is chemically bound to extracellular elements of connective tissue. However, the evidence for location of residual sodium in connective tissue (11) consists simply of finding more residual sodium at muscle ends than in their middles. This observation could be explained equally well if sodium were associated with some component of cell membranes, since these probably comprise a greater proportion (relative to volume) of the tissues at muscle ends. If the bound sodium residues outside of cells (e.g. in connective tissues) the common assumption of the uniformity of the ratio of sodium and chloride in all parts of the extracellular space will be invalidated, since chloride is freely diffusible.

In cat uteri, progesterone treatment decreases the sodium space so that it no longer exceeds, or is less than, the chloride space. This effect was not observed in the case of rabbit uteri. It is apparent that progesterone acts differently in these two species insofar as its electrolyte effects are concerned. This may be related to the difference in the effects of progesterone on the functional activities of cat and rabbit uteri (19).

The pattern of uterine sodium loss into sucrose and retention of a substantial residue was the same for all uteri, including those whose sodium space was no greater than the chloride space (cat uteri). These findings are difficult to reconcile with the common concept of the confinement of sodium to the extracellular space, when the chloride space is equal to or greater than the sodium space (15).

The two most plausible explanations for the occurrence of residual sodium in leached tissue are (*a*) chemical combination, such that residual sodium is not available for exchange, and (*b*) inhibition of a sodium "pump" and redistribution of sodium in accordance with the membrane potential. However, equilibration with isotonic potassium chloride does not affect residual sodium content in spite of the fact that it should abolish any electrical potential responsible for accumulation of sodium.

Less conclusive localization of the diffusible fraction of tissue sodium is possible. When the logarithm of the tissue sodium was plotted against time of extraction by sodium-free solutions, the results clearly did not follow a straight line, and there often appeared to be two or three separate rates of extraction. Harris and Steinbach (11) have postulated that cation diffusion from skeletal muscle cells in isotonic sucrose is dependent upon the rate of phosphate liberation from organic compounds. Phosphate extraction was not followed in the present study, but the accumulation of anions of some weak acids was indicated by an increase in pH (from 4.7 to about 6.2) and by an increase in buffering capacity. If this postulate is correct, phosphate

liberation and consequently the rate of extraction of cellular cations would be temperature dependent. While potassium extraction was temperature dependent, no appreciable fraction of tissue sodium was similarly affected. Either negligible diffusible cellular sodium was present or some independent (temperature insensitive) mechanism accounted for its extraction.[8]

3. Potassium Distribution

The potassium concentration of uterine cells appears to be at least as high as that of other tissues. In contrast to their effects on the distribution of sodium and chloride, ovarian hormones appear to exert little influence on the potassium concentration of uterine cells, although cellular growth is induced by estrogen and progesterone in this tissue. Retarded extraction of tissue potassium in potassium-free media *in vitro* as a result of reduction of temperature to 4° C. was an unexpected finding. Most tissues have been reported to lose potassium more readily at lower temperatures (15). Moreover, residual potassium after leaching in potassium-free media is higher than that reported for other tissues (15). These differences may result from differences in damage to cells in preparing pieces for *in vitro* study. Before speculation as to degrees of dependence on energy metabolism for potassium accumulation is warranted, the existence of the differences between tissues should be more rigorously established by concurrent comparisons.

4. The High Cellular Cation Content of Smooth Muscle

The uterus appears to possess a high total cellular cation concentration (200–300 meq./l. cell water, exclusive of polyvalent cation) as compared with other tissues. It seems probable that part of the cellular cation is bound in an osmotically inactive form. A high concentration of polyvalent colloidal anions in cells or connective tissues also might account for this finding. The presence of non-diffusible anions in uterine tissues also was suggested by the fact that after equilibration *in vitro* in isotonic potassium chloride, tissues had an excess of cation (10–15 meq./l.) tissue water as compared with the concentration of these ions in the extraction medium, and an even larger deficit of chloride (30–40 meq./l.) as compared with total tissue cations. The total cellular cation concentration (sodium plus potassium) was decreased by estrogen in both species by all methods of calculation, probably as a consequence of increased cellular water.

References

1. ASPER, S. P., SCHALES, O., and SCHALES, S. S. J. Biol. Chem. **168**, 779 (1947).
2. BO, W. J. and ATKINSON, W. B. Anat. Record, **113**, 91 (1952).
3. CAREY, M. J. and CONWAY, E. J. J. Physiol. **125**, 232 (1954).
4. CHEEK, D. B. and WEST, C. D. J. Clin. Invest. **34**, 1744 (1955).
5. CLARY, M. L., CAMERON, A., and CRAVER, B. N. Proc. Soc. Exptl. Biol. Med. **77**, 778 (1951).
6. CONWAY, E. J. Biochem. J. **29**, 2221 (1935).

[8] The rate of potassium loss from tissue in sucrose was about 25% lower than in experiments in which choline chloride or potassium-free saline–bicarbonate was used as the leaching medium. It would be of interest to know if phosphate emergence is the limiting factor accounting for these differences.

7. DANIEL, E. E. Ph.D. Thesis, University of Utah. 1952.
8. DANIEL, E. E. and BOYES, W. Am. J. Obstet. Gynecol. **73**, 395 (1957).
8a. DANIEL, E. E. To be published.
9. GAMBLE, J. L. and ROBERTSON, J. S. Am. J. Physiol. **171**, 659 (1951).
10. GILLMAN, J. Endocrinology, **29**, 336 (1941).
11. HARRIS, E. J. and STEINBACH, H. B. J. Physiol. **133**, 385 (1956).
12. HORVATH, B. Proc. Natl. Acad. Sci. U.S. **40**, 515 (1954).
13. KALMAN, S. M. Federation Proc. **16**, 310 (1957).
14. KNOX, W. E., AUERBACH, V. H., and LIN, E. C. C. Physiol. Revs. **36**, 164 (1956).
15. MANERY, J. F. Physiol. Revs. **34**, 334 (1954).
16. MEIGS, E. B. J. Exptl. Zool. **13**, 497 (1912).
17. NICHOLS, G. J., NICHOLS, N., NEIL, W. B., and WALLACE, W. M. J. Clin. Invest. **32**, 1299 (1953).
18. REYNOLDS, S. M. R. The physiology of the uterus. 2nd ed. P. B. Hoeber, Inc., New York, N.Y. 1949.
19. ROBSON, J. M. and SCHILD, H. O. J. Physiol. **92**, 1 (1938).
20. SCHALES, O. and SCHALES, S. S. J. Biol. Chem. **140**, 879 (1941).
21. TALBOT, N. B., LOWRY, O. H., and ASTWOOD, E. B. J. Biol. Chem. **132**, 1 (1940).
22. VAN DYKE, H. B. and CHEN, G. Am. J. Anat. **58**, 473 (1936).
23. VAN SLYKE, D. D. J. Biol. Chem. **58**, 523 (1923).
24. WALSER, W., SELDEN, D. W., and GROLLMAN, A. J. Clin. Invest. **32**, 299 (1953).
25. WILLIAMS, C. E., SEIBERT, R. A., and HUGGINS, R. A. Am. J. Physiol. **177**, 87 (1954).
26. WILSON, T. W. and BALL, E. G. J. Biol. Chem. **79**, 221 (1928).
27. YOUNG, M. K., JR. and RAISZ, L. G. Proc. Soc. Exptl. Biol. Med. **80**, 771 (1952).

EFFECTS OF VARYING PROPORTIONS OF DIETARY RAPESEED OIL ON THE RAT[1]

JOYCE L. BEARE, T. K. MURRAY, AND J. A. CAMPBELL

Abstract

Rapeseed oil, corn oil, and mixtures of the two oils containing 5, 10, 20 ,40, and 80% rapeseed oil were fed to weanling rats at levels of 10 and 20% by weight of the purified diet. A significant decrease in body weight gains and in food consumptions occurred with the two highest levels of rapeseed oil, that is, 16 and 20% by weight of the total diet. At the same levels there was an increased lipid excretion. Corn oil and rapeseed oil which had been heated to 200° C. for 120 hours produced no changes in weight gains when fed at the 10% level, but exhibited some growth-retarding effects at the 20% level. At both levels heated corn oil and rapeseed oil increased the liver weight of male rats.

The increased production of Canadian rapeseed oil and its possible incorporation into the Canadian human regimen have necessitated further study into the nutritive value of the oil. It was demonstrated by Boer (1) that rats grew less favorably on rapeseed oil than on butter, and by Deuel (6) that rapeseed oil fed as 10% by weight of the diet appreciably reduced the weight gains of young rats. Carroll (3) observed the growth-retarding action of the oil when fed as 25% by weight of the diet. In the Netherlands, Thomasson (12) showed that as the amount of rapeseed oil in the diet increased the weight gains decreased and that at the highest level of 73 Cal.% all animals died after being on diet an average of 17 days. The following study was undertaken to assess critically the extent of the growth-retarding properties of Canadian-produced rapeseed oil.

Procedure

One hundred and eighty weanling rats of the Wistar strain were divided into 18 groups similar in sex and litter distribution, and average initial body weights. They were randomly arranged in individual screen-bottomed cages. Food was supplied ad libitum, its wastage reduced by the use of screens with three squares per square inch in the feed cans, and the amount consumed measured. Corn oil, Canadian-produced rapeseed oil of the Argentine variety[2] (which has approximately 40% erucic acid in its total fatty acids), and mixtures of the two oils containing 5, 10, 20, 40, and 80% rapeseed oil were fed at levels of 10 and 20% by weight of the diet. In addition, four groups of animals were fed the basal diet with either 10% or 20% corn oil or rapeseed oil which had been heated in stainless steel beakers to 200° C. in an electric oven for 120 hours. The composition of the basal diets is shown in Table I. Food consumption and body weights were recorded twice weekly.

[1]Manuscript received July 26, 1957.
Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario.

[2]The oil was supplied through the courtesy of the Saskatchewan Wheat Pool. It had been expeller-pressed, alkali-refined, bleached, and deodorized.

TABLE I

COMPOSITION OF DIETS

Ingredients	I, g./100 g.	II, g./100 g.
Oil[a]	10	20
Cornstarch	36	30
Sucrose	24	20
Vitamin-free casein	19	19
Vitamin mixture in casein[b]	1	1
Salt mixture, U.S.P. XIV	4	4
Alphacel	6	6

[a]Oil contained 0.5 mg. menadione, 3000 I.U. vitamin D, and 15 mg. vitamin E per 100 g. diet.

[b]100 g. vitamin mixture contained 100 mg. thiamine, 100 mg. riboflavin, 100 mg. pyridoxine HCl, 300 mg. calcium pantothenate, 5 g. inositol, 500 mg. nicotinic acid, 1 g. *p*-aminobenzoic acid, 2 mg. biotin, 0.2 mg. vitamin B_{12}, 10 g. of choline chloride, 50,000 I.U. vitamin A.

A 4-day fecal collection from each rat was individually analyzed for free fatty acids and neutral lipid material by a 5-hour petroleum ether extraction in a Goldfisch apparatus. The soap content of the fecal residue was extracted with 5% acetic acid in petroleum ether in a Soxhlet apparatus for another 5 hours.

After 67 to 69 days on diet the animals were fasted overnight, anesthetized with ether, and the livers, adrenals, testes, and ovaries removed.

Differential infrared spectra of the heated oils against the respective unheated ones were determined on a Perkin–Elmer Recording Infrared Spectrophotometer, Model 21.

Results and Discussion

The weight gains, at 3, 5, and 9 weeks, of rats fed corn oil, rapeseed oil (RSO), and mixtures of the two are shown in Figs. 1 and 2. Analyses of variance were carried out and a Duncan test (8) applied wherever there were significant differences. When the oils were fed as 10% by weight of the diet there were no significant differences in weight gains. At the 20% level there was a significant reduction in weight in both sexes for the 80% rapeseed oil mixture, equivalent to 16% of the total diet, and a still further reduction for rapeseed oil as 20% of the diet. A linear response in growth retardation with increasing amounts of rapeseed oil, as indicated by the graphs, was confirmed by statistical analyses.

Food consumptions, Fig. 3, were greater with the 10% fat diets containing 4.1 Cal./g. than with the more calorific 20% fat diets containing 4.6 Cal./g. As the rapeseed oil became a more substantial part of the diet the food intake decreased.

Statistical analyses of weight gains, food consumptions, and weight gains corrected for food consumptions by covariance analyses for 3, 5, and 9 weeks

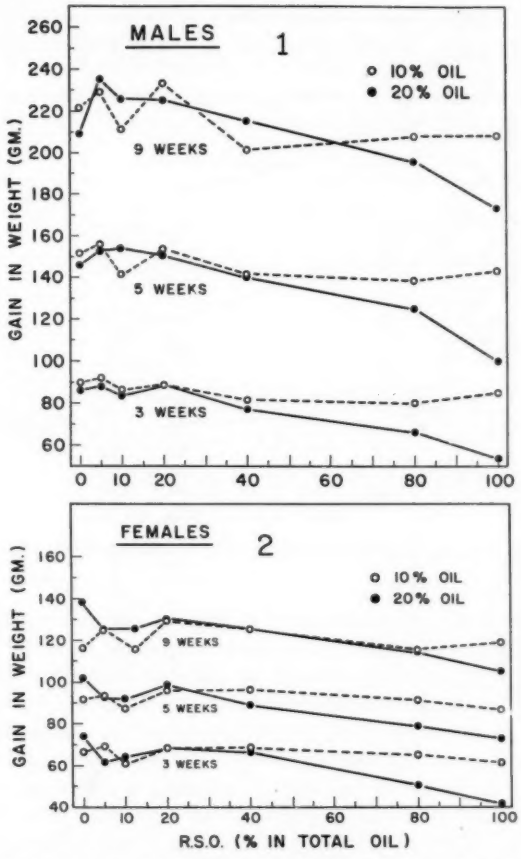


FIG. 1. Gain in weight, at intervals, of male rats fed 10 and 20% fat diets with rapeseed oil constituting 0, 5, 10, 20, 40, 80, and 100% of the total oil.

FIG. 2. Gain in weight, at intervals, of female rats fed 10 and 20% fat diets with rapeseed oil constituting 0, 5, 10, 20, 40, 80, and 100% of the total oil.

of the experimental feeding period are shown in Table II. At 7 weeks' time the same differences were significant as for 5 and 9 weeks. The high levels of oil consistently retarded the weight gains of both sexes. Since there were significant differences in the amounts of food consumed it was thought that appetite alone might be responsible for the amount of tissue synthesized. Such a finding would be in agreement with that of Thomasson (13) that the growth-retarding effect of rapeseed oil was associated with the lower food intake. However, when body weight gain was corrected for the amount of food consumed by each rat there was still a significant difference in weight gains at 3 weeks' time, indicating differences in the utilization of the diet. At 5, 7, and 9 weeks the different proportions of rapeseed oil did not affect

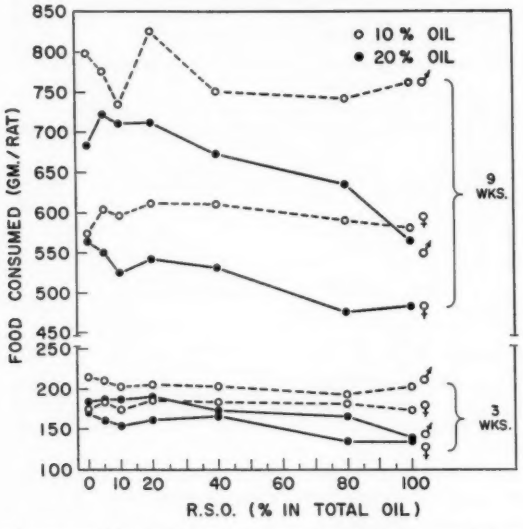


FIG. 3. Food consumed in 3 and 9 weeks by male and female rats fed varying amounts of rapeseed oil.

the corrected gains whereas the level of dietary fat did. Apparently, the animals were capable of adapting to ingested rapeseed oil after 3 weeks, and were affected by the oil only through its appetite-depressing action.

Rapeseed oil fed at the lower levels did not alter lipid excretion. At the two highest levels, as shown in Table III, it produced an increased excretion of free fatty acids and neutral lipid material. No differences were found in the soap fractions. Rapeseed oil had been shown to have a slow rate of absorption (7), and erucic acid to cause a greater lipid excretion than shorter-chain, mono-enoic fatty acids (9). Although feces were collected during the seventh week when the significant growth retardation was caused by a depressed appetite, it appeared that at least a portion of the long-chain fatty acids of rapeseed oil were unavailable for metabolism. The absolute amount of fat excreted, being relatively small in relation to the total food intake, did not significantly alter the efficiency of the diet.

In Fig. 4 total weight gains for unheated and heated corn oil and rapeseed oil are shown for 3, 5, and 9 weeks. It is interesting to note that females gained significantly more in 9 weeks on 20% corn oil than on 10% corn oil, whereas males appeared to prefer the 10% corn oil. As was the case with the unheated oils fed at the 10% level in the diet, the heated oils fed at that level did not significantly affect the gains in weight. Throughout the entire experimental period the rats receiving the cooked rapeseed oil at the 20% level had significantly lower weight gains than those receiving the same proportion of similarly-treated corn oil. At the 20% level only the females consistently distinguished between unheated and heated corn oil. The males

TABLE II

ANALYSES OF VARIANCE OF WEIGHT GAINS, FOOD CONSUMPTION, AND CORRECTED WEIGHT GAINS

	Sources of variation	Degrees of freedom	Mean squares		
			3 weeks	5 weeks[a]	9 weeks[a]
Males					
Weight gain	Fat levels	1	1320**	1152*	505
	RSO levels	6	647**	1417**	2111**
	Fat × RSO	6	298**	761**	800
	Error	56	53	221	544
Food consumption	Fat levels	1	15780**	55441**	168364**
	RSO levels	6	1350**	4686**	13500**
	Fat × RSO	6	690**	2842**	7536*
	Error	56	149	634	2488
Weight gain corrected for food consumption	Fat levels	1	254**	2504**	7842**
	RSO levels	6	45*	61	207
	Fat × RSO	6	47*	75	112
	Error	55	16	67	181
Females					
Weight gain	Fat levels	1	458**	132	111
	RSO levels	6	398**	369**	451*
	Fat × RSO	6	238**	212	312
	Error	56	50	93	176
Food consumption	Fat levels	1	10566**	30493**	90145**
	RSO levels	6	774**	1589**	3960*
	Fat × RSO	6	415*	1113*	2781
	Error	56	149	385	1427
Weight gain corrected for food consumption	Fat levels	1	227**	835**	4277*
	RSO levels	6	57*	60	41
	Fat × RSO	6	77**	50	109
	Error	55	23	53	59

[a]At 5 and 9 weeks the degrees of freedom were 51 for males and 53 for females.
*Significant at 5% level.
**Significant at 1% level.

TABLE III

FREE FATTY ACIDS AND NEUTRAL LIPIDS EXCRETED PER RAT IN 4 DAYS

RSO in diet, %	Corn oil in diet, %	Lipids, g.
0	10	0.056
10	0	0.137
0	20	0.158
2	18	0.143
4	16	0.138
8	12	0.153
16	4	0.249
20	0	0.516

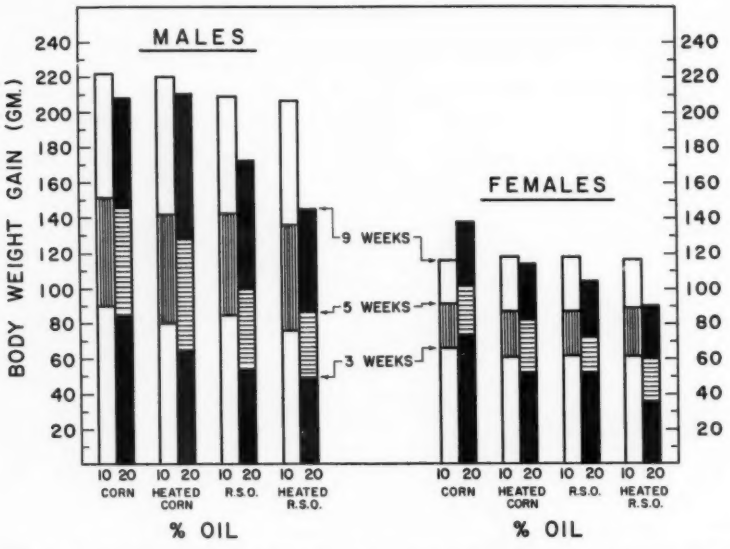


FIG. 4. Gain in weight, at intervals, of rats fed unheated and heated corn oil and rapeseed oil at levels of 10 and 20% of the diet.

eventually appeared to adapt to the heated corn oil. This differs from the findings of Johnson *et al.* (10), who fed corn oil which had been heated to 200° C. as 20% by weight of the diet to male weanling rats for 9 weeks, and at that time obtained decreased weight gains. The heating of rapeseed oil at the 20% level did not affect the males in the early part of the experiment but did later, and retarded the female weight gains at all intervals tested. Crampton *et al.* (5), who treated oil more rigorously, reported that corn oil and rapeseed oil, heat-polymerized at 275° C. for 30 hours, had a growth-depressing action when fed to rats as 10% of the diet.

The infrared absorption spectra of the heated oils showed that heating had increased the absorbance of both oils at a frequency of approximately 1725 $cm.^{-1}$, the characteristic vibration frequency for carbon–oxygen groupings, and indicated that both oils had been oxidized to the same extent. The absorption spectra also suggested the possibility that there was more polymerization in the corn oil, a not unexpected finding since corn oil contains 46–66% linoleic acid (11) compared to rapeseed oil with 17% (4).

Neither the levels of dietary fat, the proportions of rapeseed oil in the fat, nor previous heating of corn oil and rapeseed oil affected liver and adrenal weights of females. Liver weights of the males, Table IV, were increased by both heated oils, particularly corn oil. When the liver weights were corrected for respective total body weights, the standard error of the liver weight means was 0.28 g., and the effects of heating still highly significant. Dietary rapeseed oil did not affect either the absolute adrenal weights nor

TABLE IV

LIVER AND ADRENAL WEIGHTS OF MALES

Dietary fat	Level of fat in diet, %	Mean liver wt., g.	Mean adrenal wt., mg./pair
Corn oil	10	6.76	41.4
	20	6.64	43.6
Heated corn oil	10	8.36	38.6
	20	9.86	39.6
RSO	10	7.66	41.7
	20	6.27	33.3
Heated RSO	10	8.24	32.2
	20	6.60	35.7

the proportions of adrenal weight to total body weight. These findings do not agree with those of Carroll (2), who mixed the oil with Master Fox Chow and after a feeding period of 4 weeks found that rapeseed oil had increased the absolute weight of the adrenal glands. It therefore seems worthwhile to investigate the effects of the basal diet and length of feeding period on the adrenals of rapeseed-oil-fed animals.

Acknowledgments

Appreciation is expressed to Miss Constance Cox and Mrs. Eva Reimer for statistical advice and analyses respectively, to Mr. J. C. Bartlet for determinations of infrared spectra, and to Mr. C. Desloges for help in caring for the animals.

References

1. BOER, J., JANSEN, B. C. P., and KENTIE, A. J. Nutrition, **33**, 339 (1947).
2. CARROLL, K. K. Endocrinology, **48**, 101 (1951).
3. CARROLL, K. K. J. Biol. Chem. **200**, 287 (1953).
4. CRAIG, M. B. Can. J. Technol. **34**, 335 (1956).
5. CRAMPTON, E. W., FARMER, F. A., and BERRYHILL, F. M. J. Nutrition, **43**, 431 (1951).
6. DEUEL, H. J., Jr., GREENBERG, S. M., STRAUB, E. E., JUE, D., GOODING, C. M., and BROWN, C. F. J. Nutrition, **35**, 301 (1948).
7. DEUEL, H. J., Jr., HALLMAN, L., and LEONARD, A. J. Nutrition, **20**, 215 (1940).
8. DUNCAN, D. B. Biometrics, **12**, 307 (1956).
9. HOPKINS, C. Y., CHISHOLM, M. J., MURRAY, T. K., and CAMPBELL, J. A. J. Am. Oil Chemists' Soc. (In press, 1957).
10. JOHNSON, O. C., SAKURAGI, T., and KUMMEROW, F. A. J. Am. Oil Chemists' Soc. **33**, 433 (1956).
11. SNIEGOWSKI, M. S. and BALDWIN, A. R. J. Am. Oil Chemists' Soc. **31**, 414 (1954).
12. THOMASSON, H. J. J. Nutrition, **56**, 455 (1955).
13. THOMASSON, H. J. J. Nutrition, **57**, 17 (1955).

A COMPARATIVE STUDY OF THE EFFECTS OF MALEIC HYDRAZIDE AND *p*-DIMETHYLAMINOAZOBENZENE ON RAT LIVER[1]

W. A. MANNELL AND H. C. GRICE

Abstract

Rats receiving 2% maleic hydrazide (MH) in their diet and rats fed 0.06% *p*-dimethylaminoazobenzene (DAB), for periods up to 26 weeks, were compared with a control group on a stock diet. For the rats on DAB there was a decrease in body weight, in desoxyribose nucleic acid (DNA) per liver cell nucleus, and in the size of the average liver cell. There was an increase in liver weight, in DNA per liver, and in the number of cells per liver. These findings confirmed previous work with this compound. No significant changes were found in any of these measurements for the rats on MH. Pathological study revealed liver neoplasms in all animals fed DAB for 10 weeks or longer. No abnormal findings were reported for any rats on MH. The results indicate that maleic hydrazide, an antisprouting agent, does not produce any effects in rat liver similar to those caused by DAB, a known carcinogen.

Introduction

The compound known as maleic hydrazide (1,2-dihydropyridazine-3,6-dione) was introduced in 1949 as a growth regulator. Perhaps its most important application is as an antisprouting agent for potatoes and to a lesser extent, onions. When applied to potato plants 4 to 6 weeks before harvest, maleic hydrazide is translocated to the tubers and has proved effective in preventing sprouting during storage. A residue of maleic hydrazide remains in the potatoes and onions so treated and thus the safety of the chemical is of prime importance as far as its use on food crops is concerned. This is particularly important with potatoes since they comprise one of the staple foods in the diet of so many people.

In 1951 Darlington and McLeish (2) published a note on the action of maleic hydrazide on the plant cell. It was reported that it functioned as a plant growth regulator by virtue of its capacity to cause breakage of chromosomes during mitosis of the cell. The authors added that since nearly all chromosome-breaking agents have proved to be carcinogenic as well, maleic hydrazide should be suitably tested before its use was encouraged in agriculture.

Extensive investigation with animals showed that maleic hydrazide had a low order of toxicity either upon topical application or upon acute or chronic administration (8). However, the question of whether or not maleic hydrazide had carcinogenic properties still remained. The purpose of the experiments to be reported was to provide further information concerning the possible carcinogenicity of maleic hydrazide.

p-Dimethylaminoazobenzene (DAB) is an azo dye, commonly called butter yellow, which was at one time widely used as a food color. This compound has been shown to cause liver tumors when fed to rats. Thomson, Heagy,

[1]Manuscript received June 12, 1957.
Contribution from the Food and Drug Laboratory, Department of National Health and Welfare, Ottawa, Ontario.

Hutchison, and Davidson (16) fed a diet containing 0.06% DAB to rats for periods up to 6 months. They observed an increase in the total number of cells in the liver, a decrease in the average size of the cells, and a lower average content of desoxyribose nucleic acid (DNA) per nucleus in the liver cells of the treated rats. These effects occurred both in livers with obvious tumors and in livers that had not reached this stage.

Thioacetamide has also been reported as a hepatic carcinogen (6). Laird (10) gave thioacetamide to rats by subcutaneous injection and found a two-fold increase in cell number in the average liver between the 17th and 28th day of treatment. This was accompanied by a reduction in size of the average cell. There was no change in the average DNA content per nucleus.

Since these two carcinogenic compounds, DAB and thioacetamide, had similar effects on rat liver in that they both caused an increase in cell number and a decrease in average cell size, it was decided to conduct a similar experiment using maleic hydrazide to determine its effects on rat liver. A preliminary report of this work has already appeared (11).

Methods

Male rats, approximately two months old, were divided into three groups. A control group was fed the stock laboratory diet and a test group was given the same diet containing 2% maleic hydrazide in the form of the sodium salt. The third group of rats received the stock diet containing 0.06% DAB. After 6, 10, 20, and 26 weeks, six animals from each group were sacrificed by exsanguination under ether anaesthesia.

The procedure used by Thomson *et al.* (16) was followed closely. The whole liver was quickly removed, weighed, and finely chopped with scissors. A small piece of tissue was taken for histological examination, about 250 mg. were used for whole-tissue analysis, and the remainder was used for the isolation of nuclei by a modification of the citric acid procedure of Mirsky and Pollister (12). This latter portion was immediately placed in dry ice and kept frozen until the nuclei were isolated. All animals were examined for gross pathological change.

For whole-tissue analysis a combination of the methods of Schmidt and Thannhauser (14) and Schneider (15) was used. The sample was accurately weighed and homogenized in ice-cold 10% trichloroacetic acid (TCA). The homogenate was quantitatively transferred to a centrifuge tube and the extraction with TCA was continued with two additional portions. These extracts were pooled in a volumetric flask and contained the acid-soluble compounds of the tissue.

The tissue residue was then treated with a series of extractions with cold ethanol, ethanol–ether 3:1 at 70°, and ether to remove the lipid fraction. These extracts were also pooled and saved for the determination of total lipid phosphorus.

The residue was hydrolyzed in 1 *N* potassium hydroxide for 18 hours at 37°. This treatment makes possible the separation of ribose nucleic acid

(RNA) from desoxyribose nucleic acid (DNA) and permits the determination of these two compounds. By treating the hydrolyzate with cold acid, DNA is precipitated while RNA remains in solution. After the RNA fraction was removed quantitatively, the DNA was removed from the precipitate by extraction with 5% TCA at 90°. The control of temperature during this series of extractions is important. Both the RNA and DNA fractions were measured by determination of their phosphorus content using the method of King (9) except that the reducing agent was that suggested by Gomori (7). The RNA fraction, by this method of separation, contains small amounts of phosphorus not derived from RNA (3, 4), and values for RNA phosphorus (RNAP) are therefore approximate.

The procedure for the isolation of the liver cell nuclei was carried out in a cold room at a temperature of 2–4° C. The frozen liver was mixed in a Waring blendor with 1% citric acid for 6 minutes. This disrupted the cell membranes and freed the cellular contents. By a series of centrifugations and suspensions in 0.2% citric acid, and by straining the first two suspensions through finely woven cloth, a good separation of nuclei was obtained. The procedure was checked microscopically to determine the extent of the separation. The number of nuclei in the final suspension was determined by counting in a haemocytometer chamber. At least two counts were done for each sample and each was made on a separate dilution. To facilitate the nuclear count the diluting fluid used was composed of one part Delafield's haematoxylin stain to four parts water.

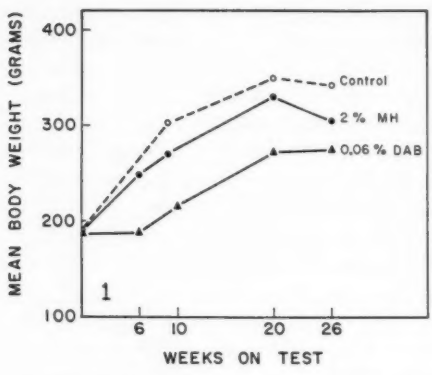
After this count had been made, an aliquot of the nuclear suspension containing a known number of nuclei was treated with one-half volume of 30% cold TCA to precipitate the nucleic acids. The procedure used for the whole-tissue analysis, slightly modified, was followed, and the DNA phosphorus (DNAP) was isolated and determined. This gave a figure for the average DNAP content per nucelus. From the chemical determinations of DNA the total amount of DNAP per liver and the total number of cells in the liver was estimated. The weight of the average liver cell was also estimated.

By this procedure, then, results were available for DNAP per nucleus, DNAP per liver, the total number of cells in the liver, and the weight of the average cell. The concentration of RNAP, lipid phosphorus, and acid-soluble phosphorus was also determined. It was felt that this would give a good comparison between the effects of maleic hydrazide and DAB.

Results

Fig. 1 shows the growth curves for the three groups of rats. The curve for the maleic hydrazide group was not significantly different from that of the control group at any of the test points. The mean body weight of the rats on DAB was significantly less than the corresponding control figures throughout the experiment.

The effect of MH and DAB on liver weight, expressed as percentage of body weight, is illustrated in Fig. 2. As in all the succeeding figures, each



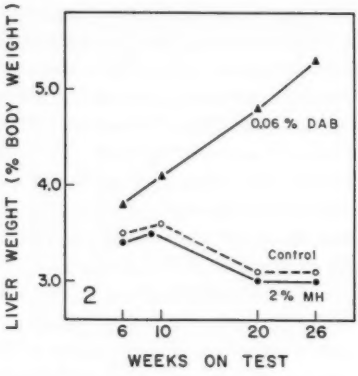


FIG. 1. Growth curves for control rats, and rats fed 2% maleic hydrazide or 0.06% DAB.
FIG. 2. Effect of administration of maleic hydrazide and DAB on rat liver weight (expressed as a percentage of body weight).

point represents the mean value for six rats except the control group at 10 weeks where three animals were used. It is apparent that liver weights for the MH group and the control group were very similar. The DAB group was significantly different from the control group after 10 weeks on test ($P<0.01$). Part of the difference was due to the lower body weight but there was an absolute increase in the weight of the liver at 20 and 26 weeks.

Results for DNA phosphorus content, expressed as mg. per liver, are given in Fig. 3. There was no difference between the values for the control group and those for the MH group at any test period. The rats on DAB showed a significant increase in the DNA content of the liver at 20 ($P<0.02$) and 26 ($P<0.01$) weeks.

This increase in DNA was not due to an increase per cell as shown in Fig. 4 where values are given for average DNAP per nucleus. It will be noted that results for the MH group were similar to those for the control group. The rats on DAB, however, showed a decrease in DNAP per nucleus after 20 weeks on test. This value was significantly less ($P<0.01$) than the control values at 6 and 10 weeks as well as the unusually high control figure recorded at 20 weeks. There was no difference recorded between the groups at 26 weeks. The value for the DAB group appeared higher than at 20 weeks and the figure for the control group was below the normal level reported for adult rat liver (about 0.9 picogram per nucleus). Thomson and co-workers (16) found a lower average DNAP per nucleus after both 5 and 6 months treatment with DAB.

Fig. 5 gives the estimates obtained for the total number of cells per liver. This is calculated by dividing the value for DNAP per liver by the value for DNAP per nucleus. Here, too, it may be seen that the results for the MH group corresponded well to those of the control group. The DAB group showed a highly significant increase in cell number at 20 and 26 weeks.

The results for tissue mass per unit weight of DNAP are shown in Fig. 6. There was no significant difference between the maleic hydrazide group and the control group. The rats receiving DAB showed a decrease in tissue mass per unit weight of DNAP which was significant as early as 6 weeks after the beginning of the experiment. Fig. 7 shows results for RNAP per unit weight of DNAP. The picture is much the same as for tissue mass, with no difference existing between control and MH groups and an early significant decrease

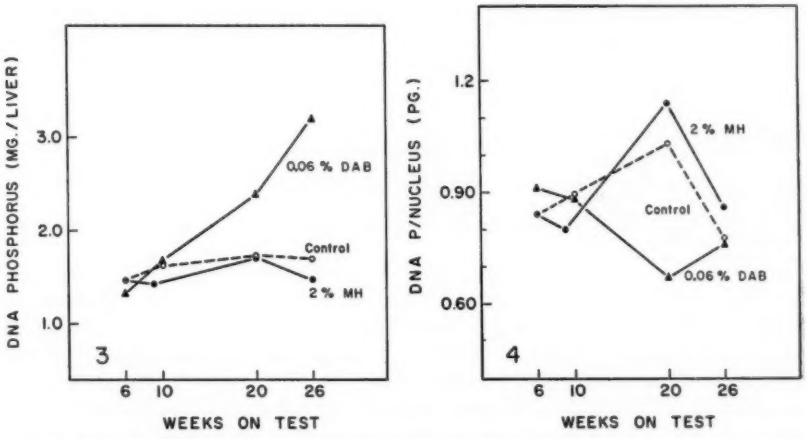


FIG. 3. Total DNA phosphorus content in livers of control rats and rats fed 2% maleic hydrazide or 0.06% DAB for times up to 26 weeks.
FIG. 4. Average DNA phosphorus per nucleus of liver cells from control rats, and rats fed 2% maleic hydrazide or 0.06% DAB; 1 picogram (pg.) equals 10^{-12} gram.

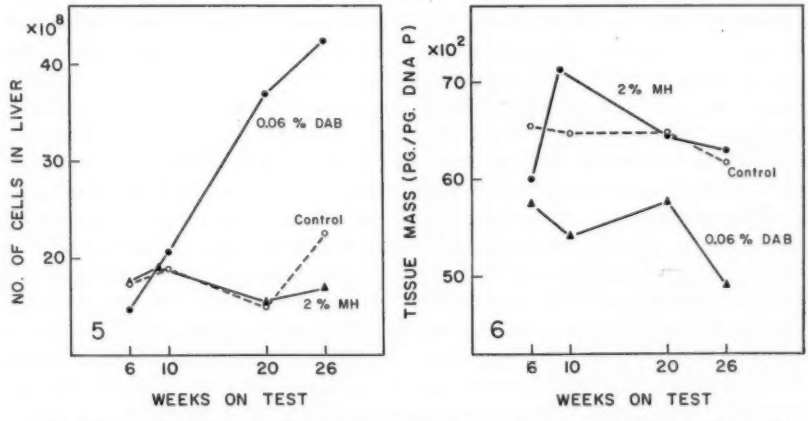


FIG. 5. Total number of cells per liver of control rats, and rats fed 2% maleic hydrazide or 0.06% DAB for times up to 26 weeks.
FIG. 6. Effect of administration of maleic hydrazide and DAB on rat liver tissue mass.

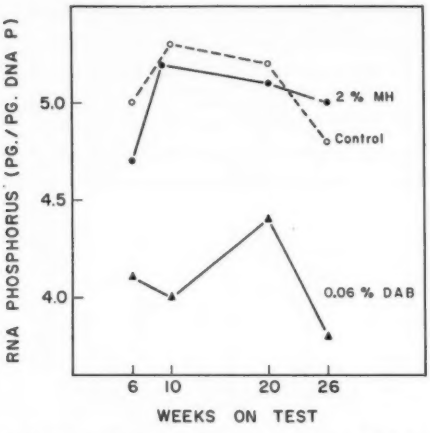


FIG. 7. Effect of administration of maleic hydrazide and DAB on RNA phosphorus content of rat liver.

for the DAB group that continued throughout the experiment. Results for lipid phosphorus (not shown) followed the same general trend. By expressing these results in terms of DNA a measure was obtained of average cell mass and average cell composition. The results indicate that there was a decrease in the average size of the liver cells of the rats receiving DAB. The fact that the average DNAP per nucleus changed during the experiment does not invalidate this method of expressing the results. These changes in tissue mass, RNAP, and lipid phosphorus would have appeared more pronounced had the average DNAP per nucleus remained constant.

A summary of the pathological findings is given in Table I. The tissues examined from the rats receiving MH showed no significant changes from those of control animals. The livers appeared normal both microscopically and macroscopically. General morphological structure was retained. Nuclear shape, size, and staining qualities of the liver parenchymal cells resembled those of control. There were no neoplasms noted in any tissue from these rats.

No liver neoplasms were observed in the rats on DAB for 6 weeks. The general microscopic morphology of the livers of these rats differed from control livers as follows: Liver cell cords were more irregular; cytoplasmic acidophilia was more varied; parenchymal cell nuclei showed a greater variation in size and some particularly large nuclei were noted. After the animals had been 10 weeks on DAB, liver neoplasms were observed. In the gross the tumors appeared in two rats as small, round, gray-white nodules. Microscopically, tumors were present in all six rats in the group. They were all carcinoma with an adenomatous pattern. Metastasis to the lung was observed in one animal. One rat had an accompanying myocarditis.

At 20 weeks small nodular neoplasms were visible in the livers of all six animals fed DAB. Microscopically the parenchymatous changes were more

TABLE I

SUMMARY OF PATHOLOGICAL FINDINGS ON RATS FED 2% MALEIC HYDRAZIDE OR 0.06% *p*-DIMETHYLAMINOAZOBENZENE

Group	Weeks on test	No. animals examined	Liver neoplasms		Remarks
			Gross	Microscopic	
Control	6, 9, 10, 20, 26	24	0	0	No abnormal findings
2% maleic hydrazide	6, 9, 20, 26	24	0	0	No neoplasms observed in any tissues
0.06% D.A.B.	6	6	0	0	No neoplasms observed
0.06% D.A.B.	10	6	2	6	The gross tumors appeared as small, round gray-white nodules. Tumors were carcinoma with adenomatous pattern; metastasis to the lung in one animal
0.06% D.A.B.	20	6	6	6	Most nodules were small. Tumors were carcinomas with adenomatous pattern
0.06% D.A.B.	26	6	6	6	Tumors were much larger and more numerous than at 20 weeks. Microscopically, car cinomas showed adenomatous and sheet patterns. Cholangiofibrosis was common

advanced than at 6 and 10 weeks. By 26 weeks the tumors were much larger and more numerous. Microscopically carcinomas showed adenomatous and sheet patterns. Cholangiofibrosis was common. The histologic characteristics of these tumors were similar to those described by Orr (13) and Edwards and White (5) in rats fed DAB. Two rats receiving DAB died between the 20th and 26th week. Death was due to massive hemorrhage from liver carcinomas.

Discussion

Thomson *et al.* (16) determined the DNA content of rat cell nuclei from relatively large numbers of animals under different conditions. A carcinogenic diet containing DAB was used as one of these conditions and produced a lower average DNAP content per nucleus in the liver than the usual figure for adult rats. The normal value for rat liver is of the order of 0.9 picograms per nucleus. This figure is higher than the value obtained for non-hepatic tissues, which is in the range 0.65–0.70 picograms DNAP per nucleus (16). The explanation given for the higher average value in liver is the presence in that organ of tetraploid and octaploid nuclei containing two and four times the amount of DNA in the usual diploid cell. According to Thomson *et al.* (16), as the number of tumor cells increases in the liver of rats fed DAB the average cell composition will approach the average composition of the tumor cells. If these cells have diploid nuclei the average DNA content per nucleus will fall, i.e. approach the value for the non-hepatic tissues.

Our results on livers of rats fed DAB are in general agreement with those of Thomson *et al.* (16). The chemical results were supported by the pathological findings. It is evident that after 20 weeks' feeding with 0.06% DAB,

tumor growth in the liver was pronounced. It might be mentioned that the basic diet used was a commercial preparation reputed to be a satisfactory maintenance diet for rats.

The results indicate that the administration of maleic hydrazide produced no changes similar to those caused in rat liver by *p*-dimethylaminoazobenzene, a known carcinogen. The maleic hydrazide was fed at a dietary level some thirty times that used for DAB.

The negative results obtained with maleic hydrazide in our experiments are in agreement with the findings recently reported by Barnes *et al.* (1). These workers conducted extensive tests over a period of 2 years in an attempt to show whether or not maleic hydrazide possessed any carcinogenic properties for mammalian tissues. These tests included feeding experiments, subcutaneous injections, skin tests in conjunction with croton oil, and tissue culture experiments. They concluded that the weight of evidence was that maleic hydrazide has no carcinogenic properties.

Acknowledgments

The authors are indebted to Naugatuck Chemical, Division of U.S. Rubber Company, for the supply of maleic hydrazide used in these experiments and for permission to refer to unpublished reports on toxicological studies of maleic hydrazide. Miss Elaine Connell, Miss Rita Carioto, and Miss Claire Beliveau rendered technical assistance.

References

1. Barnes, J. M., Magee, P. N., Boyland, E., Haddow, A., Passey, R. D., Bullough, W. S., Cruickshank, C. N. D., Salaman, M. H., and Williams, R. T. Nature, **180**, 62 (1957).
2. Darlington, C. D. and McLeish, J. Nature, **167**, 407 (1951).
3. Davidson, J. N., Frazer, S. C., and Hutchison, W. C. Biochem. J. **49**, 311 (1951).
4. Davidson, J. N. and Smellie, R. M. S. Biochem. J. **52**, 599 (1952).
5. Edwards, J. E. and White, J. J. Natl. Cancer Inst. **2**, 157 (1941).
6. Fitzhugh, O. G. and Nelson, A. A. Science, **108**, 626 (1948).
7. Gomori, G. J. Lab. Clin. Med. **27**, 955 (1942).
8. Information from Naugatuck Chemical, Division of U.S. Rubber Company.
9. King, E. J. Biochem. J. **26**, 292 (1932).
10. Laird, A. K. Arch. Biochem. Biophys. **46**, 119 (1953).
11. Mannell, W. A. and Grice, H. C. Federation Proc. **16**, 319 (1957).
12. Mirsky, A. E. and Pollister, A. W. J. Gen. Physiol. **30**, 117 (1946).
13. Orr, J. W. J. Pathol. Bacteriol. **50**, 393 (1940).
14. Schmidt, G. and Thannhauser, S. J. J. Biol. Chem. **161**, 83 (1945).
15. Schneider, W. C. J. Biol. Chem. **161**, 293 (1945).
16. Thomson, R. Y., Heagy, F. C., Hutchison, W. C., and Davidson, J. N. Biochem. J. **53**, 460 (1953).

TREATMENT OF HEMORRHAGIC SHOCK IN THE DOG WITH CHLORPROMAZINE AND/OR HYPOTHERMIA[1]

C. W. Gowdey, R. M. Kilborn,[2] and J. A. F. Stevenson

Abstract

Treatment of established hemorrhagic shock, produced in anesthetized dogs by a standardized technique, with chlorpromazine (2.5 mg./kg.) and/or hypothermia (immersion cooling: 25° C.) did not increase the survival rate and tended to shorten the survival time. The cardiac output of all the treated animals, especially those subjected to chlorpromazine combined with hypothermia, failed to return to prehemorrhage levels even after reinfusion. The results suggest that once hemorrhagic shock has occurred, treatment with chlorpromazine or hypothermia is of no value and with the combination is deleterious.

In 1952 Jaulmes, Laborit, and Benitte (20) claimed that the combination of chlorpromazine and hypothermia (28–30° C.) was useful in the prevention and probably also in the therapy of hemorrhagic shock. Others (9, 16) reported that the early administration of chlorpromazine (2 mg./kg.) increased the survival rate in the Wiggers'-type shock. Later, Overton and DeBakey (23) stated that both immediate and ultimate survival from 'irreversible hemorrhagic shock', produced by the technique of Fine (14), was improved by the exhibition before hemorrhage of hypothermia (31° C.), chlorpromazine (5 or 50 mg./kg.), or the two combined. The combination was found to be the most effective.

In this laboratory, however, the administration of chlorpromazine (5 mg./kg.) to rats after severe hemorrhagic shock had been induced failed to improve the survival rate (8). The present investigation was designed to determine whether chlorpromazine alone, or in combination with hypothermia, would improve the survival rate of dogs in which hemorrhagic shock had been already well established.

Procedure

Healthy mongrel dogs weighing between 10 and 26 kg. (mean 14.5 kg.) were used. These experiments were done in random order between the beginning of February and June. The environmental temperature ranged between 20° and 25° C. but usually did not vary by more than two degrees during a single experiment. Sodium pentobarbital, 30 mg./kg., was given intravenously and was repeated when needed. Wide-bore polyethylene catheters were placed in the left brachial and femoral arteries, and when cardiac output was to be measured, a radio-opaque catheter was introduced into the outflow tract of the right ventricle; its position was checked with a fluoroscope.

[1] Manuscript received July 23, 1957.
Contribution from the Departments of Physiology and Pharmacology, University of Western Ontario, London, Canada. The research for this paper was supported by the Defence Research Board of Canada, under Grant Number 9310-20.
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Cardiac output was determined by the Fick principle. Oxygen consumption was measured by collecting the expired air in a Tissot spirometer and passing it through a Beckman oxygen analyzer. The blood gases were determined by the method of Van Slyke.

Control measurements were begun 60 to 90 minutes after the induction of anesthesia. The pressure in the brachial artery, pulse and respiratory rates, and the rectal temperature were recorded every 10 minutes during the experimental period. Fig. 1 outlines the sequence of events in the experimental procedure.

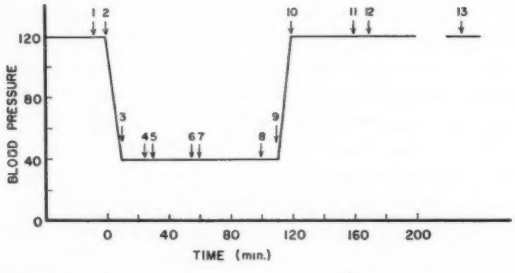

FIG. 1. Experimental protocol.

1. Prehemorrhage measurements (No. 1)*
2. 0 min.—start hemorrhage
3. 10 min.—hemorrhage to 40 mm. Hg hemobarostat on
4. 25 min.—measurements (No. 2)
5. 30 min.—reservoirs clamped off
6. 55 min.—measurements (No. 3)
7. 60 min.—chlorpromazine and/or start of hypothermia
8. 100 min.—measurements (No. 4)
9. 110 min.—start reinfusion
10. 120 min.—end reinfusion
11. 160 min.—measurements (No. 5)
12. 170 min.—start rewarming
13. Time variable—end rewarming (No. 6)

* Refers to measurements shown in Fig. 2.

The catheter in the brachial artery was attached to the monitoring manometer (set at heart level) of the 'hemobarostat' (24), and the femoral arterial catheter was connected to the blood reservoirs. This apparatus maintains a constant arterial pressure by regulating blood flow to and from the reservoirs with a maximum deviation in mean pressure of 1 to 3.5 mm. Hg; the average variation over a period of time is, however, much less. Bleeding volumes were recorded periodically from the levels in the blood reservoirs. Heparin (5 mg./kg.) was administered intravenously just before the hemorrhage, which was adjusted so that the permitted hypotensive level of 40 mm. Hg was reached in 10 minutes. This level was then maintained by means of the hemobarostat.

Thirty minutes after the beginning of hemorrhage (i.e. 20 minutes at 40 mm. Hg), the reservoirs were clamped off and the dog, now in a hypovolemic state, was allowed to regulate its own arterial pressure. Sixty minutes after the start of hemorrhage the chlorpromazine was given and/or hypothermia was begun in those animals that received treatment; 50 minutes later the blood that had remained in the reservoirs was reinfused over a 10-minute

period. When it was used, hypothermia was continued for another 60 minutes (110 minute total). Chlorpromazine (2.5 mg./kg. in 5 ml. of saline) was injected into the femoral artery over a period of 2 minutes.

Rectal temperature was measured by a thermocouple inserted 6 in. beyond the anal sphincter. The animal was supported head-up at an angle of 30 degrees from the horizontal in a canvas sling; this position was used in all experiments. Hypothermia was produced by immersing the dog to the neck in an ice bath. Detergent was rubbed into the skin before immersion and also added to the bath; a large stirrer maintained constant mixing. The temperature of the bath was held at 4° C. until the rectal temperature had fallen to 27° C. and then the bath was immediately warmed to 25° C. An attempt was made to maintain the animal at 25° C. until the end of the hypothermia period, but frequently the temperature drifted 1–2° C. When necessary, artificial respiration was provided by a Palmer pump set to deliver 150–200 ml. of room air at a rate of 16/minute using positive and negative pressure without rebreathing. To rewarm the animal the temperature of the bath was raised in 10 minutes to 45° C. where it was maintained until the rectal temperature had reached the prehemorrhage level. The bath was then drained, the catheters were removed, and the incisions closed; the animal was dried, covered with a blanket, and returned to its cage. The criterion of survival was that the dog should be alive 48 hours after the reinfusion.

The standard error of the mean is shown for important indices.

Results

Table I shows the effect of the several treatments on survival after hemorrhagic shock; all animals died before 48 hours except one control and one chlorpromazine-treated. None of the dogs in the control or hypothermia groups died during the experiment or within 5 hours of reinfusion. One animal in the chlorpromazine group showed a progressive fall in blood pressure and a steady increase in heart rate after the drug was given; it died 24 minutes later. Of the animals treated with the combination of chlorpromazine and hypothermia, three did not survive more than two hours after reinfusion. The first died within 30 minutes with a rectal temperature of 24.9° C. The chest was opened immediately, and there was no evidence of ventricular

TABLE I

EFFECT OF TREATMENT ON SURVIVAL IN HEMORRHAGIC SHOCK
NUMBER ALIVE IN EACH GROUP OF SEVEN ANIMALS

Time after reinfusion, hr.	Controls	Chlorpromazine	Hypothermia	Hypothermia and chlorpromazine
24	4	1	0	0
48	1	1	0	0
Mean survival time (hr.) of fatalities within 48 hr., and range	23 (13–44)	12 (0–22)	17 (13–23)	8 (0–13)

fibrillation; although this animal was on artificial respiration, it was our impression that inadequate respiratory exchange caused death. The second died immediately after reinfusion, with a rectal temperature of 23.4° C. In this animal ventricular fibrillation was observed. The third died at the end of rewarming; no obvious cause of death was detected.

Fig. 2 shows the effect of chlorpromazine and/or hypothermia on cardiovascular function and rectal temperature in the presence of severe hemorrhagic shock. The rectal temperature of the control and chlorpromazine-treated animals remained between 37.4 and 38.4° C. throughout, with the reinfusion of the reservoir blood at room temperature causing only a minor, temporary fall. Although the dogs under chlorpromazine and cold were about two degrees cooler than those under hypothermia alone after 20 minutes, the time taken to reach a rectal temperature of 25° C. was the same in both groups, being approximately 65 minutes. The rate of return to the prehemorrhage

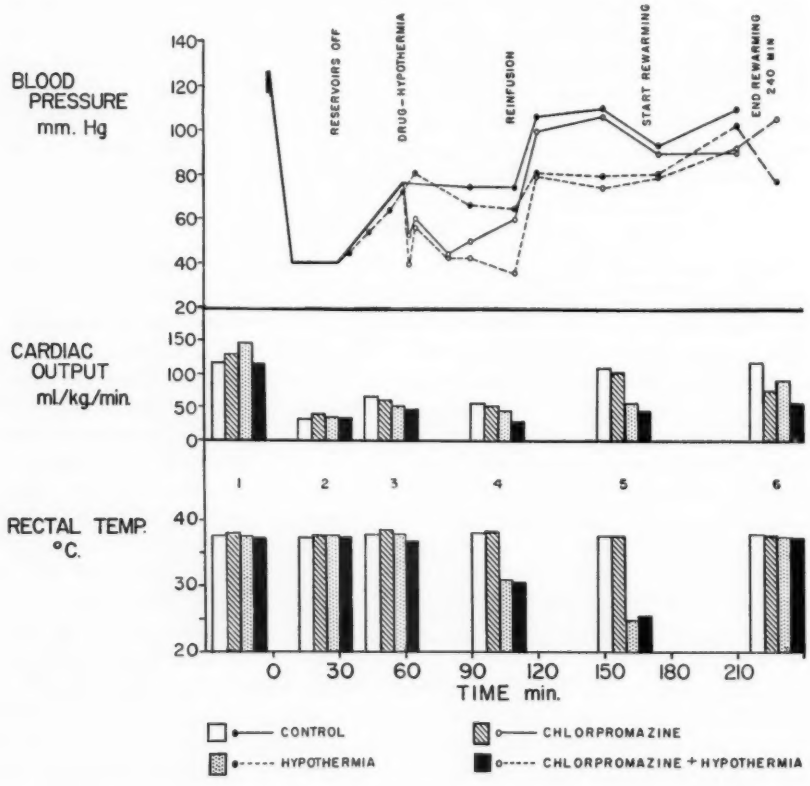

FIG. 2. Effect of chlorpromazine and/or hypothermia on cardiovascular function and rectal temperature in hemorrhagic shock.

rectal temperature was similar in the two groups with a range of 50 to 120 minutes from the start of rewarming.

To reach an arterial pressure of 40 mm. Hg in 10 minutes an average of 33.5 ± 1.75 ml./kg. of blood for the 28 animals had to be removed (initial bleeding volume). When the reservoirs were clamped off 20 minutes later, vasoconstriction had produced an average final bleeding volume of 39.1 ± 1.73 ml./kg. The animal was now allowed to regulate its blood pressure independently, and, despite the hypovolemia, every animal raised its arterial pressure during the next 20 minutes. In the controls the pressure then remained steady at a level of 74.3 ± 4.88 mm. Hg until reinfusion, when the blood pressure again rose to 105.6 ± 4.48 mm. Hg but never to the prehemorrhage level (117.1 ± 5.50 mm. Hg).

In the chlorpromazine-treated animals within 5 minutes of the start of injection of the drug, the arterial pressure dropped an average of 20 mm. Hg (8–40) and after a transient rise again dropped over the next 15 minutes. This was followed by a slow rise to 58.7 ± 7.7 mm. Hg just before reinfusion. In the hypothermia group the application of cold caused a slight rise followed by a gradual fall until at reinfusion it was 63.7 ± 4.1 mm. Hg; reinfusion and, later, rewarming produced only temporary increases. The combination of chlorpromazine and cold caused the immediate fall in arterial pressure typical of chlorpromazine, and, later, the gradual fall typical of hypothermia was exaggerated to produce a level of 35.4 ± 4.5 mm. Hg just before reinfusion.

Hemorrhagic hypotension speeded the heart; reinfusion tended to restore its rate to that before hemorrhage. Chlorpromazine caused a significant increase in the heart rate (14.3 ± 5.2 beats/minute), whereas hypothermia alone, or in combination with chlorpromazine, caused a steady decrease: at a rectal temperature of 25° C. the rate was 52.0 ± 6.1 beats/minute. After reinfusion and rewarming, the rates of all groups were similar and slightly above those before hemorrhage. In three animals of each experimental group the cardiac output was measured at various intervals. Cold alone, or in combination with chlorpromazine, markedly reduced the increase in cardiac output usually seen with reinfusion.

Hemorrhagic hypovolemia increased the total peripheral resistance, but reinfusion of the lost blood returned it to the normal range. Chlorpromazine alone had little effect, nor did it prevent the increase produced by hypothermia. Hemorrhage caused an increase from 9.8 ± 0.6 to 26.1 ± 2.2 in respiratory rate; chlorpromazine tended to enhance this response, but cold, after an initial stimulation, inhibited the response (19.4 ± 2.4/minute) as did the combination (17.4 ± 1.7/minute). At a rectal temperature of 30° C. or below, artificial respiration was necessary. On rewarming, spontaneous respirations were established when the rectal temperature reached 29–30° C.

The control group showed little change in oxygen consumption throughout the experimental period (Fig. 3) until the last determination, 210 minutes after hemorrhage, when an increase to 189 ± 34.6% of the prehemorrhage value reflected the end of anesthesia. Chlorpromazine alone increased oxygen

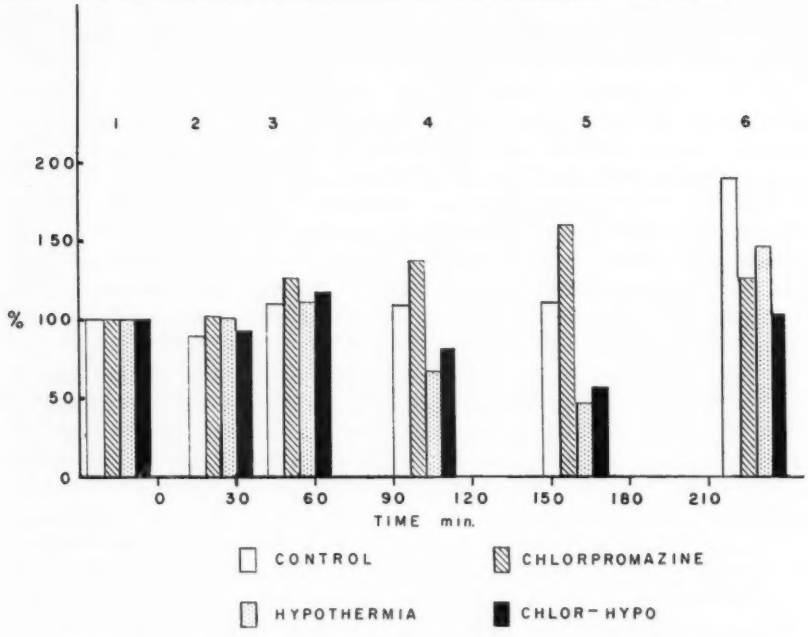

FIG. 3. Changes in oxygen consumption of the four groups throughout the experimental period. The value for each animal is expressed as a percentage of its prehemorrhage level taken as 100%, and the means of these are plotted.

consumption (to 136 ± 3.6% of the prehemorrhage value) and this increase persisted beyond reinfusion. Hypothermia reduced the oxygen consumption to 46 ± 4.9% but in those animals that received chlorpromazine as well it was reduced to 56 ± 3.5% of the prehemorrhage value.

Discussion

The effect of the shock procedure was more severe in these animals than expected. Although no animal showed spontaneous reinfusion before the reservoirs were clamped off, and all were able to increase their arterial pressure afterwards, only one control animal survived 48 hours. Nevertheless, the results clearly demonstrate that chlorpromazine in the dose used, hypothermia, or a combination of the two given after hemorrhagic hypotension are not beneficial and, in fact, shorten the mean duration of survival.

The pentobarbital anesthesia, as has been suggested (10), probably contributed to one death and to two attacks of ventricular fibrillation. Although ether has been recommended in hypothermia (15), pentobarbital was used in these experiments for reasons of safety and because our measurements have been standardized with it. It is claimed (23) that the combined use of anesthesia and hypothermia alone is followed by a mortality of 10% when antibacterial therapy is not employed.

The rectal temperature fell more quickly to 25° C. in these hypovolemic dogs than it does in normovolemic animals (7, 27), but, contrary to expectation, chlorpromazine did not shorten the total time required for cooling or for rewarming.

Axelrod (1) found that even a slight respiratory depression lowered the plasma pH more readily in hypothermia than at normal body temperature. Although the artificial respiration required during hypothermia led to an increase in the arterial content of carbon dioxide, this was only a relative increase over the low level produced by the hyperventilation of hemorrhage. Hypothermia sharply reduced the oxygen consumption; chlorpromazine had little effect. The demand for oxygen during hypothermia is stated to be reduced much more than is its availability (5, 11). The high arterial oxygen saturation that was maintained in all of the groups indicates that hypoxemia was not a contributing factor to the increased mortality in the hypothermia groups. Hemorrhage markedly increased the extraction of oxygen from the arterial blood; this had returned almost to normal at 160 minutes in the control and hypothermia groups but not in the groups treated with chlorpromazine. This reduced content of oxygen in the venous blood of all the animals that received chlorpromazine was probably due to widespread vasodilatation. The coefficient of oxygen extraction eventually rose in all groups—especially in the treated, a sign of imminent circulatory failure.

It is well known that hypothermia (2, 5, 12, 13, 18, 26) reduces the cardiac output in heart–lung preparations and in intact dogs, and Bigelow *et al.* (5) found in six out of seven dogs made hypothermic that the low cardiac output had not returned to the precooling level by 1 hour after the completion of rewarming. From our results it is presumed that treatment with chlorpromazine and/or hypothermia may lead to irreversible impairment of the already embarrassed myocardium. Whether hypothermia alone can lead to heart failure is debatable (2, 4, 5, 12, 21).

Overton and DeBakey (23) administered chlorpromazine and produced hypothermia (surface cooling: 30° C.) before the hemorrhage was begun rather than after shock had been established. Under these conditions, they found a significant protection which was most impressive with the combination of chlorpromazine and hypothermia. Chlorpromazine in its most effective dose (50 mg.), however, reduced the bleeding volumes of their animals, as it had in the experiments of Jaulmes *et al.* (20) and as the autonomic blocking agents that we found to protect against shock did when administered soon after hemorrhage (3, 22). When a blocking agent was given with no chance for reinfusion of reservoir blood to compensate for the drug-induced vasodilatation, the treated animals survived a shorter time than the controls (17). When treatment with adrenergic blocking agents or chlorpromazine was withheld, as in the present experiments, until late in the hypotensive period, no protection was afforded (8, 17, 22).

Postel, Reid, and Hinton (25), in a procedure somewhat like ours, but with a dose of pentobarbital only sufficient to prevent shivering, found that hypothermia alone (immersion cooling: 27° C.) prolonged survival but did

not affect the eventual mortality. The increase in survival time was greatest in those dogs cooled earliest in the shock sequence. Blalock and Mason (6) in 1941 had reported that the application of cold to dogs after hemorrhage did not increase the chance of survival although it lengthened the period of survival.

The present experiments indicate that the treatment of established hemorrhagic shock with chlorpromazine, hypothermia, or a combination of the two is detrimental. The reasons for this outcome can only be postulated, for the many actions and effects of chlorpromazine and hypothermia have not yet been fully investigated. The beneficial effects of chlorpromazine in preventing a deleterious response to injury are thought (19) to be due to its central action rather than to its adrenergic blocking or hypothermogenic properties. However, according to the evidence reviewed by Hopkin (19) it would not be surprising if the administration of chlorpromazine at any time after shock or damage has become established actually prejudiced the outcome. The addition of hypothermia to the treatment at this time apparently adds insult to injury.

Acknowledgments

The technical assistance of Mr. R. Payson and Mr. P. van den Hogen is acknowledged. Mr. A. G. Creed of Poulenc Limited kindly supplied the chlorpromazine (Largactil).

References

1. Axelrod, D. R. and Bass, D. E. Am. J. Physiol. **186**, 31 (1956).
2. Badeer, H. Circulation Research, **4**, 533 (1956).
3. Beck, L. C., Lotz, F., Sunahara, F. A., Gowdey, C. W., and Stevenson, J. A. F. Rev. can. biol. **13**, 50 (1954).
4. Berne, R. M. Circulation Research, **2**, 90 (1954).
5. Bigelow, W. G., Lindsay, W. K., Harrison, R. C., Gordon, R. A., and Greenwood, W. F. Am. J. Physiol. **160**, 125 (1950).
6. Blalock, A. and Mason, M. F. A. M. A. Arch. Surg. **42**, 1054 (1941).
7. Botterell, E. H., Lougheed, W. M., Scott, J. W., and Vandewater, S. L. J. Neurosurg. **13**, 1 (1956).
8. Carruthers, G. F. and Gowdey, C. W. Can. J. Biochem. and Physiol. **34**, 217 (1956).
9. Courvoisier, S., Fournel, J., Ducrot, R., Kolsky, M., and Koetschet, P. Arch. intern. pharmacodynamie, **92**, 305 (1953).
10. Covino, B. G. Am. J. Physiol. **181**, 54 (1955).
11. Delorme, E. J. Lancet, **263**, 914 (1952).
12. Edwards, W. S., Tuluy, S., Reber, W., Segal, A., and Bing, R. J. Ann. Surg. **139**, 275 (1954).
13. Evans, C. L. J. Physiol. (London), **51**, 91 (1917).
14. Fine, J. New Engl. J. Med. **250**, 889 (1954).
15. Fisher, B. A. M. A. Arch. Surg. **71**, 3 (1955).
16. Fournel, J. Compt. rend. soc. biol. **146**, 561 (1952).
17. Gowdey, C. W., Pearce, J. W., and Stevenson, J. A. F. Can. J. Biochem. and Physiol. **34**, 951 (1956).
18. Hegnauer, A. H. and D'Amato, H. E. Am. J. Physiol. **178**, 138 (1954).
19. Hopkin, D. A. B. Lancet, **268**, 605 (1955).
20. Jaulmes, C., Laborit, H., and Benitte, A. Compt. rend. **234**, 372 (1952).
21. Lombardo, T. A., Radigan, L. R., and Morrow, A. G. Circulation Research, **5**, 22 (1957).
22. Lotz, F., Beck, L., and Stevenson, J. A. F. Can. J. Biochem. and Physiol. **33**, 741 (1955).
23. Overton, R. C. and DeBakey, M. E. Ann. Surg. **143**, 439 (1956).
24. Pearce, J. W. and Gowdey, C. W. J. Appl. Physiol. **6**, 441 (1954).
25. Postel, A. H., Reid, L. C., and Hinton, J. W. Circulation Research, **5**, 594 (1956).
26. Sabiston, D. C., Jr., Theilen, E. O., and Gregg, D. E. Surgery, **38**, 498 (1955).
27. Severinghaus, J. W. and Stupfel, M. J. Appl. Physiol. **8**, 1 (1955).

THE CHROMATOGRAPHIC DETERMINATION OF GLUTAMIC, ASPARTIC, AND CYSTEIC ACIDS USING AN ANION EXCHANGE RESIN[1]

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Abstract

Optimum conditions for the separation of the acidic amino acids glutamic, aspartic, and cysteic on an anion exchange resin, Dowex-1×8, have been studied. The sample to be analyzed is adjusted to pH 7 and placed on a column of Dowex-1×8 previously equilibrated with 0.1 *M* acetate buffer at pH 4. Glutamic and aspartic acids are separated by elution with 0.1 *M* acetate buffer at pH 4.0–4.6. Cysteic acid is eluted with 0.2 *M* acetate buffer at pH 5.5–5.9. With this method quantitative separation of synthetic mixtures of the acidic amino acids has been achieved. This method has also been used to determine the dicarboxylic amino acids in acid hydrolyzates of various proteins, of normal protein-free plasma, and of normal urine.

Introduction

In 1944 Cannan (1) isolated the dicarboxylic amino acids by adsorption on Amberlite IR4, and glutamic and aspartic acids were estimated separately by isolation procedures. Kibrick (4) also used Cannan's method but he measured glutamic and aspartic acids together by a titrimetric method. Fairly large amounts of protein were used and the substances were not eluted from a column of resin according to the conventional method of chromatography.

Consden, Gordon, and Martin (2) studied the separation of acidic amino acids using Amberlite IR4. Although completely quantitative recoveries were not obtained, complete separations were achieved. Wiltshire (18) used the method of Consden *et al.* (2) to measure glutamic acid. This amino acid, however, could not be estimated by the ninhydrin method because resin decomposition products were continually eluted and hence yielded excessively high blank values. Amberlite IR-4B has been used similarly by other workers (6, 10).

Hirs, Moore, and Stein (3) recovered only 70 and 85% of aspartic and glutamic acids by elution with 0.5 *N* acetic acid from Dowex-1. Muldrey and Martinez (11) reported in an abstract the quantitative separation of glutamic, aspartic, and cysteic acids on a strong base ion exchange resin. No details of the method were available; attempts to apply the procedure were not successful, probably owing to the lack of sufficient information.

It was felt that the acidic amino acids might be recovered *in toto* by avoiding the use of acids for elution and by substituting buffers. The present investigation was initiated to develop the use of buffers for the quantitative elution and separation of glutamic, aspartic, and cysteic acids from Dowex-1×8. The buffers did not produce resin decomposition products with subsequent elution and it was, therefore, possible to analyze the amino acids in the effluent by the ninhydrin method.

[1]Manuscript received May 30, 1957.
Contribution from the Departments of Biochemistry and Pediatrics, University of Saskatchewan, Saskatoon, Saskatchewan.

Since it was only of interest in this laboratory to determine glutamic acid in various biological fluids it was felt that the very elegant and comprehensive method of Moore and Stein (7, 8) using a cation exchange resin, Dowex-50×4, was too complicated and time-consuming for our purposes. The development of the present method was intentionally restricted to the estimation of acidic amino acids. Instead of dealing with a whole spectrum of separated amino acids (7, 8), conditions have been adjusted so that the non-acidic amino acids are washed through the column in the first few milliliters of effluent, leaving only glutamic, aspartic, and possibly cysteic acid to be separated. In the method described, temperature regulation is unnecessary and very small columns are used, thus simplifying the procedure and shortening the time required to separate the acidic amino acids.

Moore and Stein (8) report that glutamine is unstable under the conditions of pH and temperature prevailing during the chromatography. Glutamine may decompose (16) in two ways depending largely on pH; the first yields pyrrolidonecarboxylic acid and ammonia, and the second gives glutamic acid and ammonia. If glutamic acid is produced to any extent by the decomposition of glutamine (14), then glutamic acid cannot be measured accurately in the presence of glutamine by such a method. In the procedure outlined here this difficulty does not arise, since glutamine is not retained on the column.

Materials and Methods

Glutamic, aspartic, and cysteic acids, crude egg albumin, crystalline egg albumin, and crystalline bovine serum albumin were purchased from Nutritional Biochemical Corporation, Cleveland, Ohio.

Glutamic acid was recrystallized three times and aspartic acid once from water. The amino acids were dried *in vacuo* over phosphorus pentoxide.

Nitrogen was determined according to the method of Ma and Zuazaga (5).

Acetate buffers were prepared as described by Peters and Van Slyke (12) according to the method given by Walpole (17).

Protein Hydrolyzates

The two crystalline proteins, egg albumin and bovine serum albumin, were dried in an oven at 105° C. overnight and then cooled and stored in a desiccator over phosphorus pentoxide. The protein (100 mg.) was hydrolyzed in a sealed tube with 30 ml. of 6 *N* hydrochloric acid at 105° C. for 48 hours. The crude egg albumin was not dried prior to hydrolysis. After it was cooled, the hydrolyzate was filtered, with washing, through a sintered glass funnel to remove insoluble humin. The filtrate and washings were concentrated to dryness *in vacuo* at 40 to 50° C. The residue was dissolved in a small amount of water and the pH was adjusted to 7 with 0.5 *N* sodium hydroxide using a Beckman pH meter, model G. The solution was then diluted with water to a suitable volume and a sample of it subjected to chromatographic analysis.

Protein-free Plasma Hydrolyzate

Mid-afternoon specimens of blood from a normal human male were collected, they were deproteinized with picric acid, and the picric acid removed

with Dowex-2×8 as described by Stein and Moore (14). To the protein-free plasma was added an equal volume of concentrated hydrochloric acid and the solution was hydrolyzed overnight under reflux. The filtration, concentration, and pH adjustment were done in the same manner as outlined for protein hydrolyzates.

Urine Hydrolyzates

Mid-afternoon specimens of urine from a normal human male were collected and hydrolyzed as described for protein-free plasma hydrolyzates.

Preparation of the Resin

Dowex-1×8* (200 to 400 dry mesh) in the chloride form was suspended in two volumes of 2 *N* sodium hydroxide. After settling, the supernatant fluid was decanted. The resin was successively washed in a similar manner with water, with 2 *N* acetic acid, and again with water. This sequence of washing with alkali, water, acid, and water was repeated three more times. Two volumes of 1 *N* acetic acid were then added to the resin and the mixture was heated on a steam-bath for 3 hours, with occasional stirring. After settling, the supernatant solution was decanted. This procedure was repeated a total of five times. Finally the wash solution was substituted by 0.1 *M* acetate buffer at pH 4 and the resin was washed on a steam-bath five times in all as described above. The resin, in a small amount of 0.1 *M* acetate buffer at pH 4, was stored at 4° C.

Preparation of the Column

A suspension of resin in 0.1 *M* acetate buffer at pH 4 was placed in a glass column with an internal diameter of 10 mm. and packed to the desired height (70 to 150 mm.) under a pressure of 20 mm. mercury. The column was equilibrated by passing through the resin about 100 ml. of 0.1 *M* acetate buffer at pH 4.0–4.6. The rate of flow was adjusted by raising or lowering the reservoir containing the buffer. After equilibration the column was ready for use.

Chromatographic Procedure

A sample at pH 6–7, varying in volume from 0.1 to 2.0 ml. and containing from 0.1 to 1.0 μM. of acidic amino acids, was allowed to flow from a pipette onto the resin. When the sample was absorbed by the resin, the surface of the resin was washed three times successively with 0.1 to 0.5 ml. portions of 0.1% BRIJ 35† in water. The detergent solution prevented the floating of fine resin particles during the washing procedure. Then 0.1 *M* acetate buffer at pH 4.0–4.6 was allowed to flow through the column. The eluate was collected in 1.0 ml. samples using a drop-counting model of the Technicon Fraction Collector. The rate of flow of the buffer through the column was adjusted to give about 4 ml. of effluent per hour. Chromatography was carried out at room temperature; no attempt was made to control the temperature.

Glutamic and aspartic acids were satisfactorily separated by 0.1 *M* acetate buffer at pH 4.0–4.6. To remove cysteic acid from the column, it was

*The Dow Chemical Company, Midland, Michigan.
†Atlas Powder Company, Wilmington, Delaware.

necessary to elute with 0.2 *M* acetate buffer at pH 5.5–5.9. The procedure was expedited by changing the buffer immediately prior to the emergence of glutamic acid in the effluent. This change in eluent also served to bring off aspartic acid more quickly, and to sharpen the peaks of both aspartic and glutamic acids. The difference in results may be seen by comparing Figs. 1 and 2. Similar differences have also been observed in many other experiments where the height of the column was the same and the concentration of amino acids was identical.

Provided that cysteic acid was not present in the sample, no ninhydrin-positive material came off after aspartic acid. Therefore, after the additional passage of 100 to 200 ml. of 0.1 *M* acetate buffer at pH 4.0–4.6, the column was ready for use again. No difference was found if the regeneration of the column was brought about with 0.1 *N* acetic acid followed by 0.1 *M* acetate buffer at pH 4. The column was rarely used more than three times in succession because of possible contamination with molds.

Analysis of Amino Acids

The contents of the tubes were developed by the modified ninhydrin method described by Moore and Stein (9). The required amount of ninhydrin reagent was made up fresh each time prior to analysis. Separate standard curves were made for glutamic, aspartic, and cysteic acids. The intensity of the color was read in a Senior Coleman, model 14.

Results and Discussion

Preliminary Observations

Initially an attempt was made to chromatograph glutamic acid according to the directions of Muldrey and Martinez (11). A sample of glutamic acid at pH 12 was placed on a column of Dowex-1×8 and eluted with 0.04 *M* sodium acetate. The results were unsatisfactory because glutamic acid came off in an extremely broad band. The high pH of the sample might have adverse effects on some free amino acids or their derivatives. Elution in three separate trials with 0.1 *M* buffers, borate (pH 9), phosphate (pH 8), and citrate (pH 6.5), resulted in the prompt stripping of glutamic acid from the column. Because of the unpromising results, elution above pH 7 was abandoned.

Consden *et al.* (2) have calculated the net charges of the acidic amino acids from pH 1 to 6. At pH 4, the differences between the net charges of glutamic, aspartic, and cysteic acids were pronounced, the respective values being −0.31, −0.69, and −0.99. Since separation depends chiefly on differences in net charge between the acidic amino acids, the expected optimum pH for resolution ought to be about 4. At this pH the affinity with which the three amino acids would be bound to the positively charged resin such as Dowex-1 should decrease in the order of cysteic, aspartic, and glutamic acid. With this theoretical background it was actually demonstrated that adequate separation of mixtures of glutamic and aspartic acids could be obtained by elution with 0.1 *M* acetate buffer at pH 4.0–4.2. Inadequate resolution was obtained with 0.1 *M* acetate buffer at pH 5.2, since the bases of the curves overlapped.

Several experiments showed, however, that elution with 0.1 *M* acetate buffer at pH 4.6 clearly separated glutamic and aspartic acids, although the curves were very close together. Elution with 0.1 *M* acetate buffer at pH 3.6 moved aspartic acid further away from glutamic acid but the aspartic acid peak was broadened. This finding tended to confirm the suggestion (2) that the pK values are depressed when these amino acids are absorbed on an anion exchange resin. Strangely enough, when 0.1 *M* citrate buffer at pH 3.1 or 0.1 *M* formate buffer at pH 2.8 was used, the dicarboxylic amino acids were not retained by the resin. It must be noted that different types of buffers were not adjusted to the same ionic strength and only acetate buffer was used at pH 4.

Additions of neutral and basic amino acids, either singly or in various combinations, to mixtures of glutamic and aspartic acids did not interfere with the separation at pH 4.0–4.6. The neutral and basic amino acids bear a net positive charge at this pH and will not be retained by a positively charged resin. Similarly, protein hydrolyzates did not hinder the resolution of dicarboxylic amino acids.

Each time a column was used over again there was gradual displacement and broadening of the curves for glutamic and aspartic acids. No apparent reason for the increasing retention of the amino acids by the resin can be offered at the present time.

Recovery of Synthetic Mixtures

After the preliminary investigations were completed, experiments were undertaken to find out if the quantitative recovery of glutamic, aspartic, and cysteic acids was complete. The results are given in Table I. Except for one figure, the recoveries are $\pm 3\%$ of theory.

TABLE I

RECOVERIES (%) OF AMINO ACIDS FROM SYNTHETIC MIXTURES

Glutamic	Aspartic	Cysteic
102.7	—	
—	100.0	
99.6	96.0	
99.0	103.1	
103.3	96.8	
98.1	100.6	
102.1	98.6	
102.2	99.0	98.2
99.4	103.2	96.5
99.1	99.6	98.6
98.8	—	—

A typical chromatographic separation is illustrated in Fig. 1. Although glutamic and aspartic acids may be separated by elution with 0.1 *M* acetate buffer at a pH of 4.0–4.6, cysteic acid is removed too slowly by this single

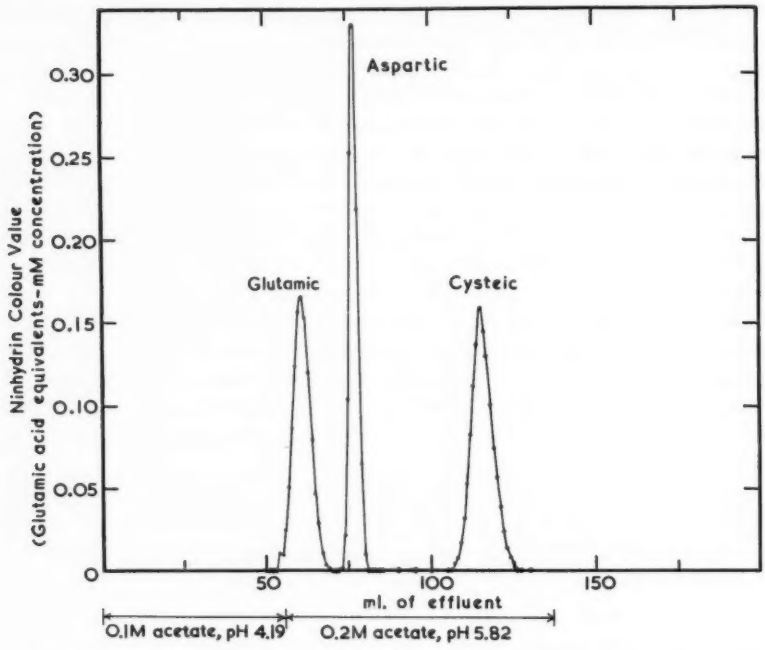

FIG. 1. Chromatographic separation of a mixture of glutamic, aspartic, and cysteic acids on Dowex-1×8 (10×70 mm.). The column of resin was equilibrated with 0.1 *M* acetate buffer, pH 4.19, and a sample, 0.3 ml., containing 3.75 mM. of each amino acid per liter was placed on the column. The pH of the sample was 6.92.

buffer solution, but, by increasing the molarity and the pH of the eluting buffer (0.2 *M* acetate at a pH 5.5–5.9), cysteic acid can be stripped off more rapidly. Provided that the height of the column is adequate, this change is also recommended for the chromatography of glutamic and aspartic acids after preliminary elution with 0.1 *M* acetate at pH 4.0–4.2. In one experiment glutamic acid was added to a urine hydrolyzate of which the glutamic acid content had been determined previously. The recovery of the added glutamic acid was 98.8%.

Analyses of Protein Hydrolyzates

The glutamic and aspartic acid contents of three hydrolyzed proteins were determined (Fig. 2) and (Table II). The peak next to the amino acid mixture (Fig. 2) was thought to be tyrosine (3) but this has not been verified. It is worthy of note that satisfactory resolution was obtained by the use of a single buffer. The acidic amino acid content of the crystalline proteins corresponds closely to the values collected by Tristram (15).

The analyses of crude egg albumin have not been compared with results in the literature because this protein was not well characterized.

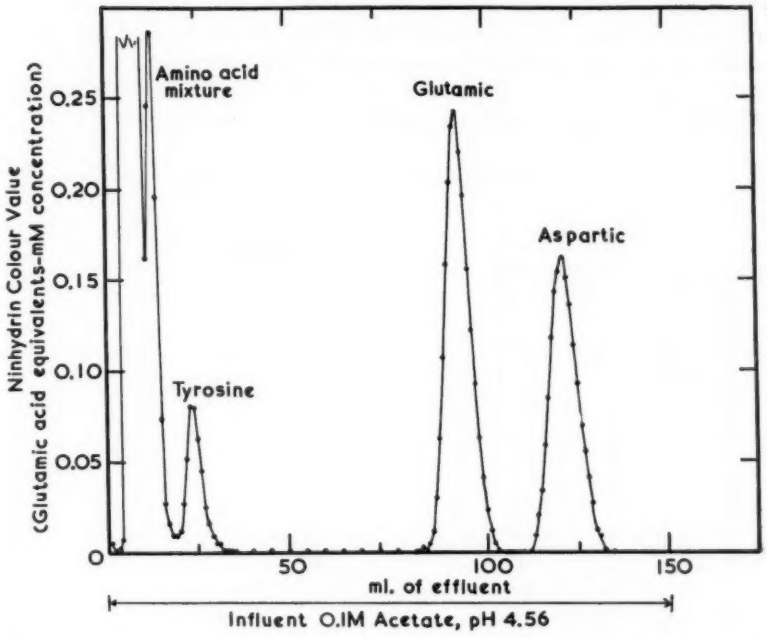

FIG. 2. Chromatographic separation of crude egg albumin hydrolyzate on Dowex-1×8 (10×150 mm.). The column of resin was equilibrated with 0.1 *M* acetate, pH 4.56. A sample of protein hydrolyzate, 0.3 ml. (=1.2 mg. of original protein) at a pH of 6.75, was placed on the column.

TABLE II

GLUTAMIC AND ASPARTIC ACID CONTENTS OF VARIOUS PROTEINS

Protein	g. N per 100 g. protein	g. glutamic per 100 g. protein	g. aspartic per 100 g. protein
Crude egg albumin	13.09	12.29	9.49
		12.19	9.03
Average		12.24	9.26
Crystalline bovine serum albumin			
	15.85	16.92	10.39
		17.09	10.61
		17.15	10.32
Average		17.05	10.44
Crystalline egg albumin			
	15.01	15.98	9.88
		15.88	9.43
Average		15.93	9.66

Analyses of Normal Protein-free Plasma Hydrolyzates

In Fig. 3 is shown the separation of a hydrolyzate of protein-free plasma. Because of the small amount of aspartic acid, it was necessary to change the eluent in order to detect this amino acid. The results are summarized in Table III. These figures approximate those reported by Stein and Moore (14).

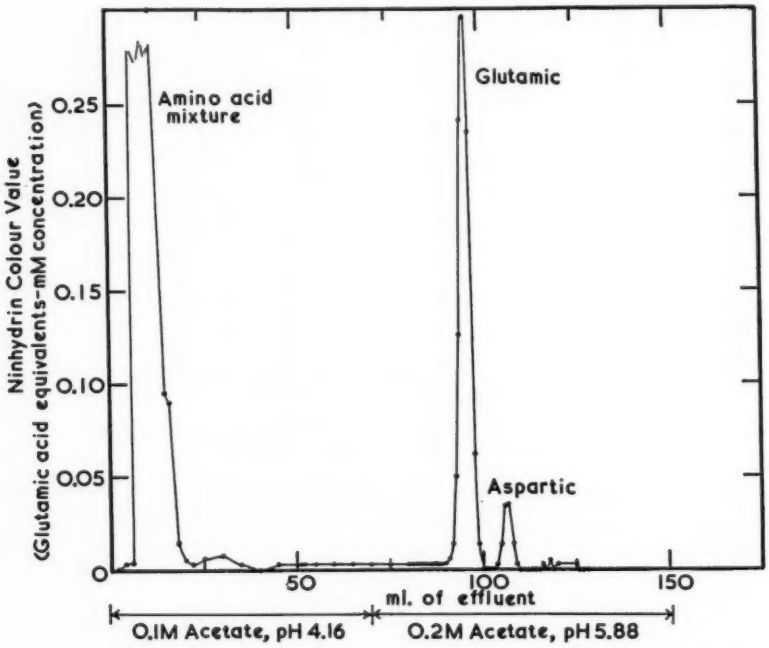

FIG. 3. Chromatographic separation of protein-free plasma hydrolyzate on Dowex-1×8 (10×150 mm.). The resin was equilibrated with 0.1 *M* acetate buffer, pH 4.16. One milliliter of hydrolyzate (=1.5 ml. of original plasma) at pH 6.29 was placed on the column.

TABLE III

GLUTAMIC AND ASPARTIC ACID CONTENTS OF NORMAL PROTEIN-FREE PLASMA AND URINE AFTER ACID HYDROLYSIS

	Glutamic acid, mg. per 100 ml.	Aspartic acid, mg. per 100 ml.
Protein-free plasma	10.41	0.9
" " "	10.23	1.0
Urine	29.2	Not done
"	29.2	" "
"	39.6	" "
"	40.3	15.4

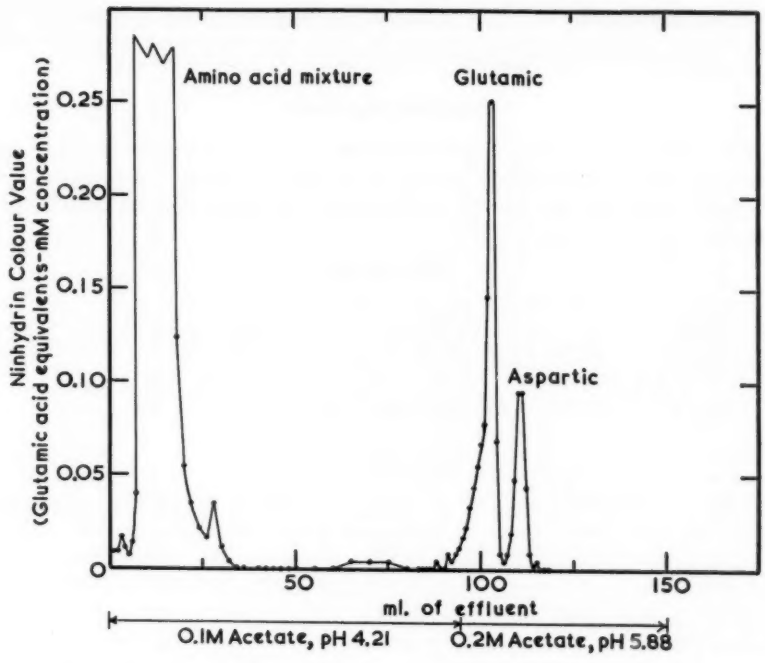

FIG. 4. Chromatographic separation of a urine hydrolyzate on Dowex-1×8 (10×150 mm.). The column was equilibrated with 0.1 *M* acetate buffer, pH 4.21. A sample, 0.3 ml. (= 0.3 ml. of original urine) at pH 6.9, was placed on the column.

Analyses of Normal Urine Hydrolyzates

Although satisfactory measurements could be obtained when protein hydrolyzates were chromatographed on short columns (about 70 mm. in height), it was found that resolution of dicarboxylic amino acids from the main amino acid mixture was poor for urine hydrolyzates under these conditions. Moreover, there was considerable spreading of the glutamic and aspartic acid peaks. These unfavorable effects were probably due to the high concentration of inorganic salts and other constituents of urine. These obstacles were easily overcome by increasing the height of the column to 150 mm. and by changing the eluent to 0.2 *M* acetate buffer at a pH of 5.88 at a suitable time. The latter modification is imperative if it is desired to estimate accurately the smaller amount of aspartic acid in urine. The chromatographic picture of a urine hydrolyzate is shown in Fig. 4.

The analytical results have been compiled in Table III. The two specimens analyzed, which had been collected at random, showed different values for their glutamic acid contents. No attempt was made to analyze 24-hour specimens because it was of no interest to determine total glutamic and aspartic acid excretions in urine but merely to indicate that the method was applicable

to such studies. The values of glutamic and aspartic acids in randomly collected urine samples are slightly higher than those reported (13) for daily excretions.

Acknowledgments

This work has been supported by a grant from the Division of Child and Maternal Health, Department of Public Health. The author is grateful to Dr. J. W. Gerrard and Dr. C. S. McArthur for their interest and helpful criticism.

References

1. CANNAN, R. K. J. Biol. Chem. **152**, 401 (1944).
2. CONSDEN, R., GORDON, A. H., and MARTIN, A. J. P. Biochem. J. **42**, 443 (1948).
3. HIRS, C. H. W., MOORE, S., and STEIN, W. H. J. Am. Chem. Soc. **76**, 6063 (1954).
4. KIBRICK, A. C. J. Biol. Chem. **152**, 411 (1944).
5. MA, T. S. and ZUAZAGA, G. Ind. Eng. Chem. Anal. Ed. **14**, 280 (1942).
6. MEIRI, S. K. and LICHTENSTEIN, N. Anal. Chim. Acta, **14**, 266 (1956).
7. MOORE, S. and STEIN, W. H. J. Biol. Chem. **192**, 663 (1951).
8. MOORE, S. and STEIN, W. H. J. Biol Chem. **211**, 893 (1954).
9. MOORE, S. and STEIN, W. H. J. Biol. Chem. **211**, 907 (1954).
10. MORRIS, P. and STUCKEY, R. E. Analyst, **78**, 636 (1953).
11. MULDREY, J. E. and MARTINEZ, W. H. Federation Proc. **15**, 686 (1956).
12. PETERS, J. P. and VAN SLYKE, D. D. Quantitative clinical chemistry: methods. The Williams & Wilkins Company, Baltimore, Md. **2**, 813 (1932).
13. STEIN, W. H. J. Biol. Chem. **201**, 45 (1953).
14. STEIN, W. H. and MOORE, S. J. Biol. Chem. **211**, 915 (1954).
15. TRISTRAM, G. R. Advances in Protein Chem. **5**, 83 (1949).
16. WAELSCH, H. Advances in Enzymol. **13**, 237 (1952).
17. WALPOLE, G. C. J. Chem. Soc. **105**, 2501 (1914).
18. WILTSHIRE, G. H. Biochem. J. **55**, 46 (1953).

STUDIES ON WHEAT PLANTS USING C^{14} COMPOUNDS

V. GERMINATION STUDIES WITH LABELLED WHEAT SEEDS[1]

W. B. McConnell

Abstract

Radioactive wheat seeds, obtained by injecting acetate-C^{14} into the stems of the parent plants, were germinated in the absence of light and nutrient and the fate of the carbon-14 was observed. Carbon respired as carbon dioxide had a higher specific activity than any of the major seed components except protein. Variations were found in the patterns by which material was transferred from the kernel to new tissue as reflected in a comparison of the activity of various components. Glutamic acid was the most active compound isolated either from the original seeds or from the new tissues. This observation, together with similarities noted in the intramolecular distribution of carbon-14 in glutamic acid of new tissue and seed residues, indicated that glutamic acid was reutilized for the biosynthesis of seedling protein. Changes in the labelling of glutamic acid during transfer to new tissue are qualitatively in accord with the idea that at least some of the amino acid is used after re-entry into the tricarboxylic acid cycle.

Introduction

Recent experiments in this laboratory on the incorporation of carbon-14 labelled compounds into growing wheat plants provided samples of mature carbon-14 labelled wheat kernels. Information about the distribution of radioactivity among the major chemical constituents of the seeds was also obtained (7), the labelling of the amino acids being studied in some detail (1, 2). It was now proposed to induce germination of these radioactive seeds. Since the seed components varied markedly in specific activity it appeared feasible to trace, at least in part, their movement into new tissue from the movement of carbon-14 during germination. Such a procedure, it was anticipated, would constitute a useful new technique for the study of certain biochemical pathways.

The present communication reports the carbon-14 content of fractions isolated from the roots, stems, and residual kernels following germination of carbon-14 labelled seeds. The results, although preliminary, are considered in terms of processes involved in material transfer from seeds to new tissue.

Experimental Methods

Carbon-14 labelled wheat seeds produced by plants administered acetate-1-C^{14} and acetate-2-C^{14} during growth were used. Details of their production and information about the mode of incorporation of the tracer into the seeds are given in previous communications (1, 2). Table I gives the distribution of carbon-14 in the kernels; the fractions collected do not account for all material, as losses of both weight and radioactivity result from discarding waste solutions (7).

[1]Manuscript received June 17, 1957.
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.
Issued as N.R.C. No. 4536.

TABLE I

DISTRIBUTION OF C^{14} IN WHEAT KERNELS FROM PLANTS INJECTED WITH ACETATE-C^{14}

Material	Acetate-1-C^{14}			Acetate-2-C^{14}		
	Weight collected, g.	Specific activity,* mμc./ mM.CO_2	C^{14}* recovered, μc.	Weight collected, g.	Specific activity,* mμc./ mM.CO_2	C^{14}* recovered, μc.
(Wheat meal)	5.00†	113	19.70‡	5.00†	144	24.80‡
Starch	2.75	66	6.37	2.65	60.2	5.80
Albumin	0.19	126	0.62	0.18	219	1.11
Gluten	0.59	234	5.88	0.61	425	11.04
Bran	0.62	77	1.75	0.57	83.6	1.79
Ether soluble	0.09	127	0.73	0.10	242	1.48
Total (exclusive of meal)	4.24		15.35	4.11		21.22
% Recovery	84.8		77.9	82.2		85.6

*Observed values for acetate-1-C^{14} have been multiplied by the factor 10/7.16, those for acetate-2-C^{14} by 10/9.8. Thus figures shown are as expected if each tracer had been administered at the rate of 10 μc. C^{14} per plant.

†Not corrected for moisture content.

‡The activity of the meal represents 38% and 48% of total activity injected into plants in the form of acetate-1-C^{14} and acetate-2-C^{14} respectively.

Growth of the Seedlings

Kernels were germinated in the absence of light and nutrient. They were placed on moist blotting paper on the plate of a desiccator modified so that a stream of carbon-dioxide-free air could be passed through it. The air entered the growth chamber after bubbling through water contained in the bottom part of the chamber. The air withdrawn from the chamber was scrubbed with dilute sodium hydroxide to collect carbon dioxide respired by the seedlings. Paper wicks, suspended through the perforations of the desiccator plate and dipping into the water, kept the seed bed moist.

To minimize the development of molds and bacteria the seeds were soaked for 1 hour in a solution of candidin (0.50 mg./ml.) and neomycin (0.02 mg./ml.) and placed thereafter in the growth chamber. The blotting paper was also treated with the neomycin solution. Gross contamination of the seeds was not observed during the tests, but when residues of the seeds were plated on a nutrient agar some viable organisms were found, almost all of which were identified as *Xanthomonas trifolii*, a common saprophyte of wheat.

Air was not passed through the chamber during the first 16 hours of the experiment, but thereafter the flow was continued without interruption; the sodium hydroxide tower for collecting carbon dioxide was changed each day. The experiments were continued until the seedlings reached an average height of about 4 inches, a period of from 5 to 7 days. The seedlings were then manually separated into "stems", "roots", and residual kernels.

Analytical Methods

Carbon dioxide respired during germination was precipitated from the alkaline solution by addition of ammonium and barium chloride (10). The

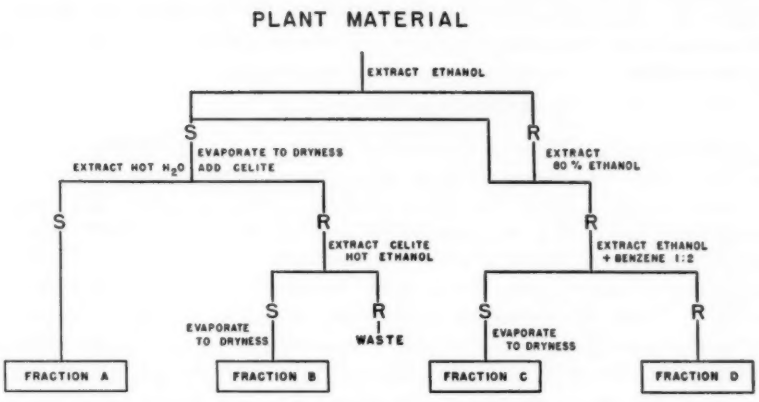

FIG. 1. Fractionation of plant material.

stems, roots, and residual kernels of the seedlings were subjected to an arbitrary fractionation scheme as outlined in Fig. 1 yielding materials soluble in water (A), ethanol (B), and ethanol–benzene 1:2 (C), and a residue (D). Some details of the procedure follow: Immediately after collection, the samples were refluxed for 15 minutes in 25 ml. of absolute alcohol. The residue was collected by filtration and washed with a few milliliters of alcohol. This step extracted much of the colored material from the plant tissue and was assumed to have inactivated enzymes. The residue was dried, ground to a fine powder, and further extracted by refluxing for 15 minutes with 80% ethanol. The combined ethanol extracts were concentrated to dryness and then thoroughly mixed with about 1 ml. of water and enough celite to form a thick paste. Twenty-five milliliters of boiling water was added and the mixture was filtered and washed with an additional 25 ml. of hot water. The water was evaporated (A). The celite was washed with 25 ml. of hot absolute alcohol and the solvent removed by evaporation (B). The residue of plant tissue not taken up in the original alcohol extractions was re-extracted under reflux with 30 ml. ethanol–benzene 1:2 (C) and the residue was dried (D). Fraction D was further fractionated by extraction of holocellulose with sodium chlorite (6). The amino acids glutamic acid, aspartic acid, phenylalanine, and tyrosine were isolated from acid hydrolyzates of another portion of fraction D by chromatography on columns of Dowex-1 (1).

The specific activity of carbon 1 of glutamic acid was obtained from carbon dioxide liberated by ninhydrin. Carbon 5 was isolated by employing the Schmidt reaction. Procedures were as previously used (1, 2). The specific activity of respired carbon dioxide was obtained by directly counting the barium carbonate collected on disks using a "micromil" end window (Nuclear Instrument and Chemical Corporation, Chicago—used in conjunction with gas flow counter D 47). Specific activity of all other materials was measured by wet combustion of the samples and counting the resulting gaseous carbon

dioxide (5). For purposes of comparison, specific activities are based on administration of the tracer to the original wheat plants at the rate of 10 μc. of C^{14} per plant.

Results and Discussion

At the onset of germination the activity of respired carbon dioxide (Table II) was lower than the activity of most kernel components (Table I) suggesting an initial release of loosely bound or adsorbed carbon dioxide. The respiration rate and specific activity increased sharply by the 3rd day and a stage was reached in which the carbon dioxide was more active than all fractions isolated except the gluten, and in acetate-2-C^{14} labelled seeds the ether soluble material. These results are in accord with the observations of Meiss (9), who working with etiolated *Lupinus albus* seedlings concluded that there is a normal but obligatory utilization of seed protein as respiratory substrate. The gluten did not appear to be the only important contributor to respired carbon dioxide, however, for, with a group of kernels which had been labelled by feeding glycine-C^{14} (8), the specific activity of carbon dioxide reached a level of 140 mμc./mM. CO_2 in contrast with a value of only 57 mμc./mM.CO_2 for the gluten. "Albumin", the other protein fraction of the original seeds, was the only known constituent as active as the respired carbon dioxide.

The results may be accounted for most readily by assuming that glutamic acid of seed proteins contributes significantly to respiratory carbon dioxide. The specific activity of glutamic acid isolated from all samples of seeds was greater than that of respired carbon dioxide (that of glutamic acid from gluten of kernels labelled with glycine-1-C^{14} was 198 mμc./mM.CO_2; see also Table IV). No simple numerical relation could be established with the present data between the specific activity of the respired carbon dioxide and that of the glutamic acid or any single carbon in the glutamic acid. It is clear, however, that starch respiration cannot account for the activity of carbon dioxide given off during germination.

TABLE II

CARBON DIOXIDE-C^{14} FROM LABELLED SEEDLINGS

	Acetate-1-C^{14}*		Acetate-2-C^{14}†	
Day	Weight recovered, mg.	Specific activity, mμc./mM.CO_2	Weight recovered, mg.	Specific activity, mμc./mM.CO_2
1	21	53	14	93
2	55	166	37	218
3	155	200	62	231
4	201	165	75	224
5	200	180	92	224
6			118	214
7			82	197
Total weight	632		480	

*76 labelled kernels germinated (wt. 2.36 g.).
†72 labelled kernels germinated (wt. 2.0 g.).

TABLE III

YIELDS OF FRACTIONS FROM SEEDLING PARTS

Material	Yield, % of original seedling weight		
	Kernels	Stems	Roots
Seeds injected with acetate-1-C^{14}			
Water soluble (A)	11.7	8.1	4.2
Ethanol soluble (B)	1.6	1.0	0.9
Ethanol–benzene soluble (C)	0.3	0.1	0.1
Residue (D)	26.4	8.1	10.8
Total	40.0	17.3	16.0
Seeds injected with acetate-2-C^{14}			
Water soluble (A)	11.9	6.4	4.1
Ethanol soluble (B)	1.8	0.8	0.8
Ethanol–benzene soluble (C)	0.3	0.2	0.2
Residue (D)	31.0		

Table III gives the yields of material isolated from the major parts of the young plants. The bulk of the dry matter remained in the kernel and the new tissue (stems and roots) accounted for little more than 30% of the original kernel weight. Part of the original seed carbon (16 or 17%) was released as carbon dioxide as indicated by comparing the weight of respired carbon dioxide with the carbon dioxide obtained by wet combustion of the wheat meal (5). The largest proportion of the kernel residue after germination was insoluble but almost one half of the shoots was extractable with water.

Notable variations were found in the patterns by which material was transferred from the kernel to new tissue, as reflected in a comparison of the activity of various fractions and other components such as holocellulose and several amino acids (Table IV). In some instances, most noticeably with fractions B and C, weakly labelled carbon was selectively utilized for the generation of new tissue. In others, the stem and root components had greater activity than corresponding components in the residue of the seeds. Furthermore, differences in the intramolecular distribution of carbon-14 in seed constituents appear to be reflected by labelling patterns in new tissue. This is suggested by differences in the mode of distribution of carbon-14 in new tissue arising from kernels labelled with acetate-1-C^{14} and acetate-2-C^{14}. Thus water-soluble material in kernels, stems, and roots obtained from seeds labelled via acetate-1-C^{14} is of about equal activity, whereas the corresponding fraction of new tissue produced during germination of acetate-2-C^{14} labelled seeds is more active than in the parent kernel material. Other examples of pattern variations are to be found in the labelling of holocellulose and of the amino acids aspartic acid, phenylalanine, and tyrosine.

As in the original seeds, glutamic acid was the most radioactive substance separated, and although dilution of activity had occurred it appears that glutamic acid in the seedling tissues was to a considerable extent derived from glutamic acid of the seed protein. Little dilution in aspartic acid activity was

TABLE IV

SPECIFIC ACTIVITY OF SOME COMPOUNDS ISOLATED FROM SEEDLINGS

Material	Specific activity ($m\mu c./mM.CO_2$)		
	Kernels	Stems	Roots
Seeds labelled with acetate-1-C^{14}			
Water soluble (A)	87	81	87
Ethanol soluble (B)	100	39	38
Ethanol–benzene soluble (C)	103	5	5
Residue (D): Holocellulose	64	64	64
Glutamic acid	458 (619)*	230	250
Aspartic acid	148 (144)	143	140
Phenylalanine	60 (50)	68	70
Tyrosine	68 (70)	80	133
Seeds labelled with acetate-2-C^{14}			
Water soluble (A)	107	146	151
Ethanol soluble (B)	249	111	95
Ethanol–benzene soluble (C)	41	2	3
Residue (D): Holocellulose	70	84	70
Glutamic acid	882 (840)	600	530
Aspartic acid	362 (357)	441	343
Phenylalanine	69 (56)	86	81
Tyrosine	82 (73)	89	97

*Values in parenthesis are those for the amino acids isolated from the gluten protein of seeds prior to germination.

observed and, on the contrary, when this acid was isolated from the stems of seedlings produced by kernels labelled with acetate-2-C^{14} it was significantly more active than in the original seed tissue. This increase in activity and the absence of apparent dilution in other samples of aspartic acid could be explained by assuming that the aspartic acid in "new" protein came in part from glutamic acid, possibly by re-entry of the latter into the Krebs cycle. The uniformity of labelling of aspartic acid from kernels, stems, and roots from acetate-1-C^{14} labelled kernels thus cannot be considered as indicating that protein aspartic acid does not mix with any radioactive pool that is either more or less radioactive.

To obtain further information about reutilization of glutamic acid the specific activities of carbons 1 and 5 were determined (Table V). A reflection of the original distribution of carbon-14 is evident in glutamic acid of stems and roots. The changes that have occurred are in qualitative agreement with the idea that a significant amount of transported glutamic acid re-entered the tricarboxylic acid cycle (TCA cycle) prior to incorporation into new protein. If glutamic acid were to re-enter the TCA cycle as α-ketoglutaric acid it could undergo decarboxylation to succinate and subsequently appear as oxalacetate (3). Oxalacetate, upon condensation with acetyl coenzyme A, could progress through the cycle to appear again as α-ketoglutaric acid, the latter would re-form glutamic acid or recycle again,

TABLE V

DISTRIBUTION OF CARBON-14 IN GLUTAMIC ACID FROM SEEDLING PARTS

	Per cent of total activity in glutamic acid			
	Acetate-1-C^{14}		Acetate-2-C^{14}	
	1-C	5-C	1-C	5-C
From gluten of original seeds	20 (620)*	63 (1950)	8.0 (340)	4.8 (200)
Kernel residue after germination (Fraction D)	22.4 (520)	64 (1470)	7.6 (330)	5.1 (220)
Stems (Fraction D)	29.0 (330)	42 (480)	12.4 (370)	7.8 (230)
Roots (Fraction D)	27.8 (350)	45 (560)	12.7 (340)	6.4 (170)

*Values given in parenthesis are the observed specific activities expressed as $m\mu c./mM.CO_2$.

each recycling resulting in the elimination of carbon 1 and one half of each of carbons 5 and 2 as carbon dioxide. The remaining carbon of positions 5 and 2 would appear in position 1.

Glutamic acid labelled by feeding plants acetate-1-C^{14} (specific activities of carbons 1, 2, 3, 4, and 5 are in the proportion of approximately 16:3:2:2:50 (2)) would on recycling lose a large part of its radioactivity, and the activity of carbon 1 compared to that of carbon 5 would increase, depending upon the amount of reutilized glutamic acid that had recycled. These are in fact the observations made (Table V). According to the same scheme smaller dilutions of carbon-14 in glutamic acid labelled predominantly in the internal carbons would be expected from recycling. The results with kernels labelled via acetate-2-C^{14} are again in qualitative agreement (specific activities of carbons 1 to 5 in glutamic acid are in the proportion of approximately 5:12:11:27:3 (2)).

It is evident from the results presented that glutamic acid of seed protein is in part reutilized to supply glutamic acid of new tissue protein. It appears moreover that this may involve re-entry of some glutamic acid into the TCA cycle. It is altogether probable that the amide of glutamic acid, glutamine, plays an important role in the results observed (4). The experimental techniques used, which involve hydrolysis by strong acids, convert the amide to the free acid, and thus no information was obtained concerning the glutamine in the plant tissues. Detailed interpretation of the present data has not been attempted but it appears that more information significant to a consideration of probable processes occurring during germination could now be obtained by similar investigations using seeds containing a very different distribution of carbon-14. As details are learned about the way carbon-14 is distributed through seed constituents following injection of different tracers, it should be feasible to interpret germination experiments more fully, in terms of biochemical processes. This technique may, therefore, provide an effective new approach to certain problems in plant biochemistry.

Acknowledgments

The author is indebted to Dr. A. C. Blackwood for identification of the organism *Xanthomonas trifolii*, to Dr. W. A. Taber for supplying candidin used in its control, to Mr. J. Dyck for determination of specific activities, and to Mr. M. Mazurek for able technical assistance.

References

1. Bilinski, E. and McConnell, W. B. Can. J. Biochem. and Physiol. **35**, 357 (1957).
2. Bilinski, E. and McConnell, W. B. Can. J. Biochem. and Physiol. **35**, 365 (1957).
3. Black, A. L. and Kleiber, M. Biochim. et Biophys. Acta, **23**, 59 (1957).
4. Bonner, J. Plant biochemistry. Academic Press, Inc., New York. 1950. p. 294.
5. Buchanan, D. L. and Nakao, A. J. Am. Chem. Soc. **74**, 2389 (1952).
6. Jermyn, M. A. Modern methods of plant analysis. Vol. II. *Edited by* K. Paech and M. V. Tracey. Verlag von Julius Springer, Berlin. 1955. p. 197.
7. McConnell, W. B. and Ramachandran, L. K. Can. J. Biochem. and Physiol. **34**, 180 (1956).
8. McConnell, W. B. Unpublished data.
9. Meiss, A. N. The formation of asparagine in etiolated seedlings of *Lupinus albus* L. The Connecticut Agricultural Experiment Station, New Haven, Bull. 353. 1952.
10. Neish, A. C. Analytical methods for bacterial fermentation. Natl. Research Council Can. Rept. No. 46-8-3. 1952.

INFLUENCE OF VITAMIN D ON CALCIUM RESORPTION AND ACCRETION[1]

B. B. MIGICOVSKY

Abstract

The absorption of calcium through the intestinal mucosa of the chick has been shown to come under the influence of vitamin D within 2 to 4 hours after vitamin D administration.

A technique is described for the measurement of calcium accretion and resorption rates in the toe bone of a chick. The effect of vitamin D on the rates of accretion and resorption was determined, and it is concluded that vitamin D affects the accretion rate, which in turn could bring about an increase in the resorption rate per bone. It cannot be stated whether the action of the vitamin D on the bone is direct or indirect.

Introduction

It is argued that the mechanisms of intestinal absorption of calcium and/or the mechanism of bone deposition are the primary targets for vitamin D. Investigations to date have provided only indirect evidence for these contentions. A direct primary effect of the vitamin on either the intestinal epithelium or the bone has not been demonstrated to the satisfaction of many investigators.

The dramatic but not necessarily direct effect of vitamin D on calcium absorption has been observed repeatedly. Patrick (7), using the method of Migicovsky and Nielson (5), observed the calcium absorption effect within 4 hours of vitamin D administration. This observation provides further indirect evidence for the claim that calcium absorption is a primary effect of vitamin D. Increased calcification is, of course, the important physiological response to vitamin D, but there is doubt as to whether it is a primary response. Bauer *et al.* (1), as a result of their studies on the movement of Ca^{45} in the rat, concluded that vitamin D increases the resorption rate of bone and that this accounts for higher plasma levels of calcium and phosphorus and increased accretion rates of bone salt.

The experiments outlined in this paper confirm the dramatic calcium absorption effect of vitamin D. Measurements of skeletal accretion and resorption in toes of chicks lead us to a different interpretation, with respect to the action of vitamin D on bone, from that adopted by Bauer *et al.* (1).

Short Term Effect of Vitamin D on Calcium Absorption

Methods

The Ca^{45} method of measuring relative absorption as described by Migicovsky and Nielson (5) was used in this study. Migicovsky and Jamieson (4) showed that, within 15 minutes of oral administration of Ca^{45}, sufficient

[1]Manuscript received February 26, 1957.
Contribution No. 343, Chemistry Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.

isotope accumulated in the bone to permit measurement of differences due to vitamin D. This fact enabled us to determine how long after a single dose of vitamin D the calcium absorption effect can be observed.

In the present study 1-day-old chicks were taken at random and fed the rachitogenic diet of the Association of Official Agricultural Chemists. When the chicks were 3 weeks of age the feed was removed and, after they had been starved for 24 hours, 0.5 ml. water or 0.5 ml. of a colloidal suspension of vitamin D (5000 units) in water was administered orally. At specified intervals following the dose of water or vitamin D the chicks were given an oral dose of Ca^{45} (2.2 mg. and 2×10^6 c.p.m.) and were killed at specified intervals thereafter. The tibiae were removed and analyzed for Ca and Ca^{45}. Details of the experiment and the results are shown in Table I. Each value represents the mean of four groups of five chicks per group.

TABLE I

TIME LAPSE OF VITAMIN D EFFECT ON CALCIUM ABSORPTION

Interval between doses of vit. D or H_2O and Ca^{45}, min.	Interval between dose of Ca^{45} and removal of tibia, min.	Mean % Ca^{45} dose per tibia	
		Vit. D	No vit. D
40	40	0.19	0.16
120	40	0.30	0.16
40	120	0.63	0.55
120	120	0.97	0.59**

**Effect of vitamin D significant at $P=0.01$.

Results

When 40 minutes are allowed to elapse between vitamin D and Ca^{45} administration, the effect of the vitamin is not significant, irrespective of the lapse of time between Ca^{45} dosage and tibia removal. The effect of vitamin D is significant when 2 hours is the time lapse between vitamin D and Ca^{45} administration. The effect is greater when the time lapse between Ca^{45} dosage and tibia removal is 2 hours.

Therefore the influence of vitamin D on the absorption mechanism is apparent only after 2 to 4 hours have elapsed. It has yet to be demonstrated that the skeletal response occurs as early.

Measurement of Calcium Resorption and Accretion Rates

It is difficult to explain the changes in bone wrought by vitamin D as due solely to an effect on the amount of calcium absorbed through the intestinal mucosa. Migicovsky and Emslie (2) showed that the rachitogenic properties of a diet for chickens could be overcome by altering the level of calcium and phosphorus. Although many levels and ratios of Ca and P were tried, no diet was devised which did not show some improvement in the rachitic lesion (% bone ash) when vitamin D was added.

Bauer *et al.* (1), in studies with the rat, concluded that physiological quantities of vitamin D have a stimulating effect on bone resorption or dissolution. In their opinion this resorption effect is of primary importance in the phenomena of bone salt accretion. Migicovsky and Emslie (3) concluded, as a result of calcium excretion studies, that vitamin D prevented the dissolution of bone salt. Migicovsky and Nielson (6) showed that implanted bone was dissolved to a greater extent when implantation was made in a vitamin-D-deficient host.

In view of these discrepant findings with respect to the effect of the vitamin on bone resorption, an attempt was made to study this aspect more directly.

Methods and Results

The values which enter into a calculation of accretion and resorption rates, such as mg. Ca/g. bone and specific activity of bone calcium, exhibit large interchick variation. Therefore, where the planned variable is time and different chicks are used, the effect of the planned variable may be obscured by a high error variance. To overcome this difficulty, a biopsy technique was introduced into the design of the experiment, so that each chick was its own control with respect to the time variable.

One-day-old chicks were taken at random and fed a rachitogenic diet. After 1 week the chicks were given an oral dose of Ca^{45} (1,725,000 c.p.m. and 1.39 mg.). When they were 3 weeks of age, one-half of the chicks were given 5000 units of vitamin D_3 per day for 4 days. When they were 3 weeks and 4 days of age, eight groups (five birds per group) each of treated and untreated chicks were sampled and the two terminal phalanges of the middle toe, left foot, were biopsied. Four days later, the same toe of the right foot was removed and blood was taken at the same time. The toe bones were analyzed for Ca and Ca^{45} in groups of five and the left and right toes were paired off, i.e., the left toe bones of five chicks were compared with the right toe bones of the same five chicks. In this manner interchick variation for the time variable was minimized.

The data obtained from the above analyses included:

total radioactivity (c.p.m.) of the left toe at time t_1,
total radioactivity (c.p.m.) of the right toe at time t_2,
specific activity (c.p.m./mg. Ca) of the left toe at time t_1,
specific activity (c.p.m./mg. Ca) of the right toe at time t_2,
mg. Ca per left toe at time t_1,
mg. Ca per right toe at time t_2,
specific activity (c.p.m./mg. Ca) of serum Ca at time t_2.

The methods used in the determination of the above values were as described by Migicovsky and Nielson (5). The data are presented in Table III.

Computation of Accretion and Resorption

The difference in the amount of calcium between the left and the right toes (ΔCa) represents the resultant of Ca accretion and resorption during the time interval between t_1 and t_2, i.e.,

[1] $$Ca_A - Ca_R = \Delta Ca$$

or

[2] $$Ca_A = \Delta Ca + Ca_R;$$

Ca_A = accreted calcium in terms of mg. Ca,

Ca_R = resorbed calcium in terms of mg. Ca.

Similarly, the difference in total radioactivity (c.p.m.) between the left and right toes (Δ c.p.m.) represents the resultant of c.p.m. accreted and resorbed during the time interval between t_1 and t_2, i.e.,

[3] $$\text{c.p.m.}_A + \text{c.p.m.}_R = \Delta\,\text{c.p.m.};$$

c.p.m._A = c.p.m. accreted,

c.p.m._R = c.p.m. resorbed.

Equation [3] may be rewritten

[4] S.A. of accreted Ca $\times$ Ca_A − S.A. of resorbed Ca $\times$ Ca_R = Δ c.p.m., where S.A. = specific activity in terms of c.p.m./mg. Ca. We can insert the value for Ca_A from equation [2] into equation [4] and get

[5] S.A. of accreted Ca(ΔCa+Ca_R) − (S.A. of resorbed Ca $\times$ Ca_R) = Δ c.p.m., which becomes

[6] Ca_R(S.A. of accreted Ca − S.A. of resorbed Ca) = Δ c.p.m. − (S.A. of accreted Ca $\times$ ΔCa)

and

[7] $$Ca_R = \frac{\Delta\,\text{c.p.m.} - (\text{S.A. of accreted Ca} \times \Delta\,\text{Ca})}{\text{S.A. of accreted Ca} - \text{S.A. of resorbed Ca}}.$$

It appears that if we could determine the values for the specific activities of accreted and resorbed calcium during the time interval between t_1 and t_2, we could arrive at a value for the amounts of calcium accreted and resorbed during the time interval under consideration.

Approximation of the Specific Activity of Resorbed Calcium

The assumption is made that in a chick, at any time within 3 weeks after Ca^{45} dosage, the specific activity of the calcium being resorbed from a particular bone is equal to the specific activity of the bone. Therefore, if the change in specific activity of the toe bones during the time interval under consideration can be considered linear, the specific activity of the resorbed calcium may be expressed by the equation

[8] $\frac{1}{2}$ (S.A. of bone at t_1 + S.A. of bone at t_2) = S.A. of resorbed Ca.

The linearity of the change in specific activity of bone calcium over short periods of time is shown in Figs. 1 and 2. In this experiment chicks were on the same diet as described above. They were dosed orally with Ca^{45} and two groups of four chicks per group were sampled at different times. They were bled and their tibiae were excised. The specific activities of the bone calcium and serum calcium were determined according to the methods described by Migicovsky and Nielson (5). The results (Figs. 1 and 2) indicate that over short periods of time, especially at the end of the experiment, which is the period under consideration, there is a negligible departure from linearity.

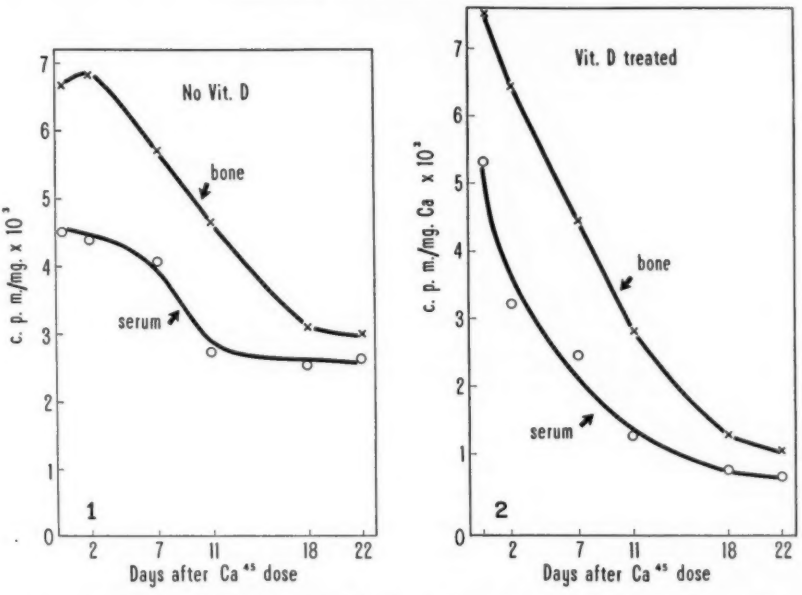

FIG. 1. Specific activity of calcium in tibia and serum at intervals after Ca^{45} dosage. Each point represents the mean of two groups of four chicks per group. The chicks were 3 days old when the Ca^{45} was orally administered.

FIG. 2. Specific activity of calcium in tibia and serum at intervals after Ca^{45} dosage. The vitamin D was placed in the diet 3 days after Ca^{45} oral administration (chicks 6 days old). Each point represents the mean of two groups of four chicks per group.

It is felt, therefore, that the above method of calculating the specific activity of resorbed calcium yields a reasonable approximation.

Approximation of the Specific Activity of Accreted Ca

The assumption is made that at any time t, the specific activity of the calcium being accreted is the same as that of the serum calcium. Therefore, if the change in specific activity of serum calcium is linear during the interval t_1 to t_2, the specific activity of the accreted calcium may be expressed by the equation

[9] $\frac{1}{2}$ (S.A. of serum Ca at t_1 + S.A. of serum Ca at t_2) = S.A. of accreted Ca.

The linearity of the change in specific activity of serum calcium over short periods of time is shown in Figs. 1 and 2. Especially is this the case in the later time intervals.

In the experiments which are conducted for the purpose of calculating accretion and resorption rates, we do not determine the specific activity of serum calcium at time t_1. The reason is that the specific activity of the serum calcium is quite low at this stage and more blood than we could safely draw is required for a determination. Therefore, we must approximate the value for the specific activity of serum calcium at time t_1.

TABLE II

RATIO OF SPECIFIC ACTIVITY OF CALCIUM IN SERUM AND TIBIA AT INTERVALS AFTER Ca^{45} DOSAGE[a]

Age of chick, days	(S.A. of serum Ca)/(S.A. of tibia Ca)	
	Vit. D treated	No vit. D
6	.71	.70
8	.50	.66
13	.55	.71
17	.46	.60
24	.57	.82
28	.62	.88

[a]Ca^{45} dose was given when the chicks were 3 days of age and vitamin D treatment began when they were 6 days of age. Each value represents the mean of two groups of four chicks per group.

It has been observed, as shown in Table II, that when different groups of chicks are killed at different times the ratio (S.A. of serum calcium)/(S.A. of bone calcium) does not change significantly. That is to say, the variance with time is no greater than the variance between groups at any one time. Visual examination of the data will show that in the later stages, which is the time interval under consideration, the variation with time is obviously not significant. It is perhaps pertinent to observe that if the ratio does not change significantly with respect to time in an experiment where different groups of chicks are used at different times, certainly it may be assumed that in any one chick the ratio is constant over a short interval of time, 20 to 24 days after the Ca^{45} dose.

If the above assumption regarding constancy of the ratio with a 4-day period is reasonable, we can calculate the specific activity of serum calcium at time t_1 from the equation

$$[10] \qquad \frac{\text{S.A. of serum Ca at } t_1}{\text{S.A. of bone Ca at } t_1} = \frac{\text{S.A. of serum Ca at } t_2}{\text{S.A. of bone Ca at } t_2},$$

which becomes

$$[11] \qquad \frac{\text{S.A. of serum Ca at } t_2 \times \text{S.A. of bone Ca at } t_2}{\text{S.A. of bone Ca at } t_2} = \text{S.A. of serum Ca at } t_1.$$

Substitution of the value for S.A. of serum Ca at t_1 in equation [9] gives

$$\frac{1}{2}\left(\frac{\text{S.A. of serum Ca at } t_2 \times \text{S.A. of bone Ca at } t_1}{\text{S.A. of bone Ca at } t_2} + \text{S.A. of serum Ca at } t_2\right)$$

$$[12] \qquad = \text{S.A. of accreted Ca.}$$

Equation [12], left side, is the same as

$$[13] \qquad \frac{\text{S.A. of serum Ca at } t_2}{\text{S.A. of bone Ca at } t_2} \times \text{S.A. of resorbed Ca.}$$

By means of the reasoning described above, the data shown in Table III were used to calculate the rates of accretion and resorption of calcium in the

TABLE III

DATA ON BONE AND BLOOD SERUM

Group[a]	Total activity per toe, c.p.m.		Specific activity		Ca per toe, mg.		Serum S.A.,
	t_1[b]	t_2	t_1	t_2	t_1	t_2	t_2
			Vit. D untreated				
1	9245	9639	2492	2434	3.71	3.96	1984
2	8866	8577	2456	2318	3.61	3.70	1677
3	9932	10028	2212	2152	4.49	4.66	1800
4	11379	11301	2888	2763	3.94	4.09	2238
5	10412	10192	2837	2682	3.67	3.80	1878
6	10116	10894	2882	2779	3.51	3.92	2207
7	9681	10799	2262	2105	4.28	5.13	1507
8	9570	9919	2473	2373	3.87	4.18	1754
			Vit. D treated				
9	12458	13580	1501	1286	8.30	10.56	694
10	11362	12444	1767	1474	6.43	8.50	888
11	15859	16286	2010	1501	7.89	10.85	768
12	12870	13444	1405	1160	9.16	11.59	686
13	13538	14268	1535	1331	8.82	10.72	750
14	12830	13243	1620	1282	7.92	10.33	694
15	14506	14570	1867	1401	7.77	10.40	717
16	10874	11700	1504	1220	7.23	9.59	550

[a]Each group value represents a pooled sample from four chicks.
[b]The time interval between t_1 and t_2 was 4 days.

toe bone of vitamin-treated and vitamin-deficient chicks. An example of the calculation is as follows:

c.p.m. in left toe = 9245 c.p.m. in right toe = 9639

Δc.p.m. = +394

mg. Ca in left toe = 3.71 mg. Ca in right toe = 3.96

ΔCa = + 0.25 mg.

S.A. of Ca in left toe = 2492
S.A. of Ca in right toe = 2434
S.A. of Ca resorbed = $\frac{1}{2}$ (2492 + 2434) = 2463

S.A. of serum Ca at t_2 (when right toe was removed) = 1984

$$\frac{\text{S.A. of serum Ca at } t_2}{\text{S.A. of Ca in right toe}} = \frac{1984}{2434} = 0.815$$

S.A. of Ca accreted = 2463 × 0.815 = 2007

Insertion of these values into equations [1] and [3] gives

$$Ca_A - Ca_R = 0.25 \text{ mg.}$$
$$2007\ Ca_A - 2463\ Ca_R = 394 \text{ c.p.m.}$$
$$Ca_R = 0.24 \text{ mg.}$$
$$Ca_A = 0.49 \text{ mg.}$$

Time lapse between left and right toe removal = 4 days.

Therefore Ca_R per day = 0.06 mg.

Ca_A per day = 0.12 mg.

The results of the calculation for the experiment are summarized in Table IV.

TABLE IV

INFLUENCE OF VITAMIN D ON THE RATE OF ACCRETION AND RESORPTION OF CALCIUM

	Vitamin D		No vitamin D	
	Mg. per toe	Mg. per 100 mg. toe Ca	Mg. per toe	Mg. per 100 mg. toe Ca
Calcium resorption per day	0.44**	4.84	0.13	3.17
Calcium accretion per day	1.04**	11.41**	0.19	4.66
Net increase of calcium per day	0.60**	6.57**	0.07	1.49
Calcium content	9.14**		4.01	
Accretion rate / Resorption rate	2.57*		1.68	

*Effect of vitamin D significant at $P = 0.05$.
**Effect of vitamin D significant at $P = 0.01$.

Discussion

It is apparent that in the presence of vitamin D the absolute quantity of resorbed Ca per day per bone is greater than in the absence of vitamin D. The accretion rate and difference between accretion and resorption is also greater in the presence of vitamin D. Bauer *et al.* (1) found a similar effect for vitamin D in rats. On the other hand, when values for Ca_R and Ca_A and net increase of Ca are expressed as per cent of calcium in the bone, the effect of vitamin D on the resorption rate is not significant. The effect on the accretion rate and the net increase is highly significant. It will appear, therefore, that there is no valid evidence to show that vitamin D is primarily and directly associated with resorption. If vitamin D does exert a direct effect on the bone, it is more likely that it is associated with the accretion rate. It is my contention that vitamin D increases the calcium accretion rate of bone. An increased resorption rate in terms of Ca per bone would follow if the normal shape of the bone were maintained. The net effect, in the presence of vitamin D, is always a gain in calcium by the bone.

The question as to whether this increased movement of calcium is a direct and primary effect of the vitamin on the bone or due to an increased flow of calcium to the bone is still moot. It does not appear likely, however, that resorption of bone calcium is under the direct influence of vitamin D. Certainly if the rate of bone growth is increased and the shape of the bone remains relatively unchanged, the absolute amount of resorption in terms of Ca per bone must be greater. This can hardly constitute a direct effect of the vitamin. In any case, resorption in terms of mg. resorbed/mg. bone Ca is not affected by vitamin D.

Acknowledgment

The advice of Dr. A. R. G. Emslie and the technical assistance of A. G. Ward, J. A. Kennedy, and L. J. Carter is gratefully acknowledged.

References

1. BAUER, G. C. H., CARLSSON, A., and LINDQUIST, B. Kgl. Fysiograf. Sällskap. i Lund Förh. **25**, 1 (1955).
2. MIGICOVSKY, B. B. and EMSLIE, A. R. G. Arch. Biochem. **13**, 195 (1947).
3. MIGICOVSKY, B. B. and EMSLIE, A. R. G. Arch. Biochem. **20**, 325 (1949).
4. MIGICOVSKY, B. B. and JAMIESON, J. W. S. Can. J. Biochem. and Physiol. **33**, 202 (1955).
5. MIGICOVSKY, B. B. and NIELSON, A. M. Arch. Biochem. Biophys. **34**, 105 (1951).
6. MIGICOVSKY, B. B. and NIELSON, A. M. Science, **115**, 354 (1952).
7. PATRICK, H. and SCHWEITZER, G. K. Poultry Sci. **34**, 832 (1955).

ABNORMALITY OF ESTROGEN METABOLISM IN HUMAN SUBJECTS WITH MYOCARDIAL INFARCTION[1]

WILLIAM S. BAULD, MORRIS L. GIVNER,[2] AND IAN G. MILNE[3]

Abstract

Estrogen metabolism has been investigated in male subjects with and without previous myocardial infarction. The urinary excretion of estriol, estrone, and estradiol-17β has been measured 1 day before and 4 days after intramuscular injection of estradiol-17β. The excretion of the individual estrogens resulting from the administration of estradiol was determined by subtracting preinjection values from the daily excretion following injection. The resultant values of estriol (T), estrone (O), and estradiol-17β (D) were expressed as the following ratios:

$$\Delta \frac{T}{O} \text{ and } \Delta \frac{T}{O+D}.$$

These urinary estrogen ratios were found to be significantly higher in subjects with previous myocardial infarction than in control subjects. The ratios in the infarction group were not influenced by bed rest nor by the duration of time following infarction which varied from 1 week to 2 years.

Introduction

Myocardial infarction is rare in women before the menopause. Oliver and Boyd (20) in a study of 1000 consecutive patients with clinical and electrocardiographic evidence of myocardial infarction or ischemia found that coronary artery disease is eight times more frequent in men than in women under the age of fifty and that this sex difference decreases markedly in the older age group. Adlersberg, Schaefer, Steinberg, and Wang (1) state that coronary artery disease in subjects under the age of fifty occurs about nine times more frequently in men than in women but that the proportionate number of females with this disorder increases after the menopause. The decrease with age in the preponderance of men over women in the incidence of coronary artery occlusion is also borne out by the pathological studies of Schlesinger and Zoll (29). These data suggest a protective action of the functioning ovary.

Previous studies on the mechanism of this protective action have mainly been confined to the effect of estrogens on plasma lipids because of the relationship between abnormalities of plasma lipids and coronary artery disease (12, 17). The concentrations of plasma lipids (21) and the urinary estrogen excretion (5) both vary during the normal menstrual cycle. Moreover,

[1]Manuscript received June 27, 1957.
Contribution from the Department of Metabolism and the McGill University Clinic, The Montreal General Hospital, Montreal 25, Canada. Presented in part at the 19th Meeting of the Canadian Physiological Society, London, Ontario, October 13, 1955. Published in part in abstract form in the proceedings of the 48th Annual Meeting of the American Society for Clinical Investigation, J. Clin. Invest. **35**, 689 (1956). This study was supported by the National Research Council of Canada, Charles E. Frosst and Co., Montreal, Eli Lilly and Co., Indianapolis, and Ayerst, McKenna, and Harrison Ltd., Montreal.
[2]Rebecca and Solomon Salmon Memorial Research Foundation Fellow.
[3]R. Samuel McLaughlin Foundation Research Fellow.

TABLE I

DIAGNOSIS AND AGE DISTRIBUTION OF SUBJECTS STUDIED

Control group			Myocardial infarction group			
Subject	Clinical status	Age	Subject	Site and duration of infarction		Age
IL	Normal subject	48	MAC	Posterior infarction	10 days	60
EG	Pulmonary emphysema	68	SA	Anterolateral infarction	4 weeks	67
RO	Normal subject	28	MCL	Posterior infarction	2 weeks	68
WA	Peripheral atherosclerosis	69	WI	Anterior infarction	10 days	50
ME	Pneumococcal pneumonia	70	HA	Anterior infarction	2 weeks	63
RA	Normal subject	39	DE	Posterior infarction	4 weeks	66
BE	Coarctation of the aorta	25	WY	Anterior infarction	4 weeks	52
YA	Mitral stenosis—commisurotomy	51	PAR	Anterior infarction	1 week	52
FO	Rheumatoid arthritis	63	TH	Anterior infarction	4 weeks	36
MIL	Normal subject	32	SM	Anterolateral infarction	4 weeks	59
BA	Normal subject	38	RE	Anterior infarction	4 weeks	55
WE	Bronchogenic carcinoma	81	FR	Anterior infarction	2 years	63
AR	Duodenal ulcer	26	CO	Posterior infarction	2 weeks	42
PO	Interstitial pulmonary fibrosis	55	REO	Anterior infarction	4 weeks	55
WIM	Pulmonary emphysema	58	MAR	Posterior infarction	11 weeks	41
SH	P.T.A. deficiency*	61	HO	Posterolateral infarction	4 weeks	64
MI	Normal subject	35	PAQ	Posterior infarction	2 weeks	57
HAN	Gastric ulcer	64	GH	Anterior infarction	4 weeks	60
CH	Hemoptysis of unknown origin	44	LA	Anterior infarction	4 weeks	41
BR	Pulmonary emphysema	62	ST	Anteroseptal infarction	4 weeks	69

*Plasma thromboplastin antecedent deficiency.

abnormal concentrations of plasma lipids and lipoproteins in male subjects with myocardial infarction are corrected by administration of ethinyl estradiol (22). The effect of estrogens in the prevention of recurrent myocardial infarction is now being studied in a number of centers.

The present investigation is a different approach to the problem of the relative immunity to coronary artery disease of women before the climacteric. The availability of a specific method of known sensitivity, accuracy, and precision for the determination of urinary estrogens (2) has made possible a study of estrogen metabolism in human subjects. In order to avoid difficulties arising from changes in the endogenous output of estrogens, which occur to a marked extent during the menstrual cycle and may occur to a lesser extent after the cessation of menses, this initial study has been confined to males. Two groups of men, with and without clinical and electrocardiographic evidence of previous myocardial infarction, have been studied. The urinary excretion of estriol, estrone, and estradiol-17β was determined before and after the parenteral administration of estradiol-17β.

Material

Selection of Cases

Myocardial Infarction Group

These patients showed the typical clinical syndrome of myocardial infarction and a Q wave pattern in the electrocardiogram indicative of transmural infarction. None had congestive cardiac failure and all showed normal liver and kidney function by the standard biochemical tests. The time of initial study following infarction varied from 1 week to 2 years (Table I). In four cases (SM, FR, MAR, ST) there had been more than one episode of myocardial infarction. Patients studied within the first 4 weeks following infarction were on complete bed rest and anticoagulant therapy. The individual ages are shown in Table I. The mean age of the group was 56.2 years (S.E. 2.1).

Control Group

The control subjects consisted of six healthy laboratory personnel and 14 hospital in-patients free from hypertensive-vascular and coronary artery disease. The electrocardiogram in each case was normal. The clinical status of the in-patients of this group is shown in Table I. Ambulant and bed patients were included. The therapeutic regimes of the in-patients were continued during these studies. The mean age of the control group was 50.9 years (S.E. 3.7). The individual ages are shown in Table I. The mean age of the control group is not significantly different from that of the infarction group.

Experimental

Procedure

A 24 hour specimen of urine was collected (Control, Day 1). Estradiol-17β (350–500 μg. in 0.4 ml. of peanut oil) was then injected intramuscularly and

urine collected for this and the subsequent three 24-hour periods (Days 2, 3, 4, 5). Each specimen was analyzed in duplicate for estriol, estrone, and estradiol-1β by the method of Bauld (2).

Calculations

The amounts of estradiol-17β, estriol, and estrone excreted were converted to the following ratios to show the relative quantitative significance of these three urinary metabolites.

1. Endogenous estriol/estrone (T/O)

$$\frac{T}{O} = \frac{\mu\text{g. of estriol excreted on Day 1}}{\mu\text{g. of estrone excreted on Day 1}}$$

2. Endogenous estriol/estrone + estradiol ($T/O+D$)

$$\frac{T}{O+D} = \frac{\mu\text{g. of estriol excreted on Day 1}}{\mu\text{g. of estrone and estradiol excreted on Day 1}}$$

3. Endogenous estrone/estradiol (O/D)

$$\frac{O}{D} = \frac{\mu\text{g. of estrone excreted on Day 1}}{\mu\text{g. of estradiol excreted on Day 1}}$$

4. Exogenous estriol/estrone, $\triangle\frac{T}{O}$, is the ratio of increase of estriol to increase of estrone resulting from the injection of estradiol-17β.

$$\triangle\frac{T}{O} = \frac{\text{estriol excreted on Days 2, 3, 4, 5} - 4(\text{estriol excreted on Day 1})}{\text{estrone excreted on Days 2, 3, 4, 5} - 4(\text{estrone excreted on Day 1})}$$

5. Exogenous estriol/estrone + estradiol, $\triangle\frac{T}{O+D}$, is the ratio of increase of estriol to increase of estrone and estradiol-17β resulting from the injection of estradiol-17β.

$$\triangle\frac{T}{O+D} = \frac{\text{estriol excreted on Days 2, 3, 4, 5} - 4(\text{estriol excreted on Day 1})}{\text{estrone + estradiol excreted on Days 2, 3, 4, 5} - 4(\text{estrone + estradiol on Day 1})}$$

6. Exogenous estrone/estradiol, $\triangle\frac{O}{D}$, is the ratio of increase of estrone to increase of estradiol-17β resulting from the injection of estradiol-17β.

$$\triangle\frac{O}{D} = \frac{\text{estrone excreted on Days 2, 3, 4, 5} - 4(\text{estrone excreted on Day 1})}{\text{estradiol excreted on Days 2, 3, 4, 5} - 4(\text{estradiol excreted on Day 1})}$$

7. Percentage recovery is the percentage of administered estrogen appearing as estriol, estrone, and estradiol-17β in the urine during the 4 days following injection.

$$\%\text{ recovery} = \frac{\triangle T + \triangle(O+D)}{\text{estradiol injected}}$$

Results

Fig. 1A shows the typical pattern of estrogen excretion during the experimental period in a control subject (RA). Before injection (Day 1) the 24 hour urine specimen contained 5 μg. of estriol, 3.5 μg. of estrone, and 2 μg. of estradiol-17β. Estrone and estradiol-17β reached a maximum excretion on the day of injection (Day 2) but the maximum excretion of estriol was delayed until the day after the injection (Day 3). The excretion of all three estrogens returned to preinjection levels by the fifth day of the experiment. The increases above control levels occurring in response to the intramuscular injection of estradiol-17β (500 μg.) were 37 μg. for estriol, 39 μg. for estrone, and 16 μg. for estradiol-17β. The recovery of injected estrogen as estriol, estrone, and estradiol-17β was therefore 18%. The increase in excretion of estriol was approximately two-thirds that of estrone and estradiol combined and approximately equal to that of estrone alone.

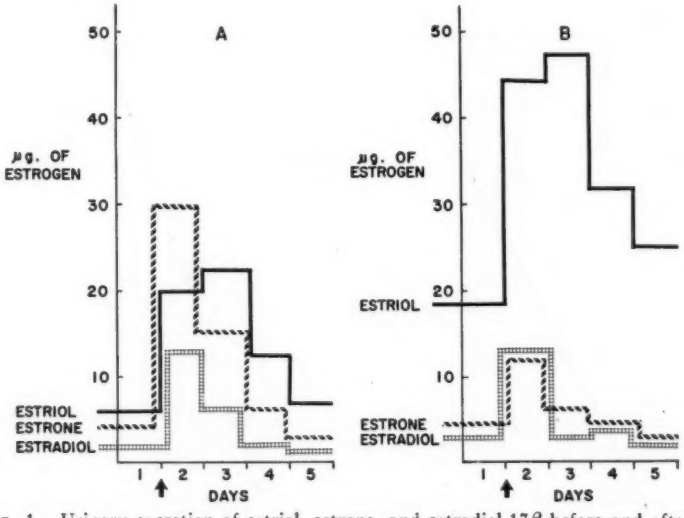

FIG. 1. Urinary excretion of estriol, estrone, and estradiol-17β before and after the injection (↑) of estradiol-17β in a control subject (A) and in a patient with previous myocardial infarction (B).

Fig. 1B shows the typical pattern of estrogen excretion during the experimental period in a case (MAR) of myocardial infarction. Before injection (Day 1), the 24 hour urine specimen contained 17 μg. of estriol, 4 μg. of estrone, and 3 μg. of estradiol-17β. Again estrone and estradiol-17β reached a maximum excretion on the day of injection (Day 2) and the maximum excretion of estriol was delayed until the day after injection (Day 3). The excretion of estrone and estradiol-17β returned to preinjection levels by the fourth day of the experiment but estriol was still elevated by the fifth day. The increases above control levels occurring in response to the intramuscular injection of estradiol-17β (410 μg.) were 75 μg. for estriol and 10 μg. for

estrone and for estradiol-17β. The recovery of injected estrogen as estriol, estrone, and estradiol-17β was therefore 23%. The increase in excretion of estriol was approximately 3.5 times greater than that of estrone and estradiol combined and approximately 7.5 times greater than that of estrone alone.

The patterns of estrogen excretion (Fig. 1) resulting from the administration of estradiol-17β are typical of the two groups. Fig. 2 shows the data obtained on the individual subjects. The increments in excretion of estriol ($\triangle T$), estrone plus estradiol ($\triangle O+D$), and estrone ($\triangle O$) were calculated from the total excretion and the endogenous level, as described in "Methods". In Fig. 2A, $\triangle$ estriol is plotted against $\triangle$ (estrone + estradiol). In Fig. 2B, $\triangle$ estriol is plotted against $\triangle$ estrone. While two of the 'control' subjects and three of the 'myocardial infarction' patients show anomalous patterns, there is a clear difference in the relative amounts of estrogens excreted by the two groups.

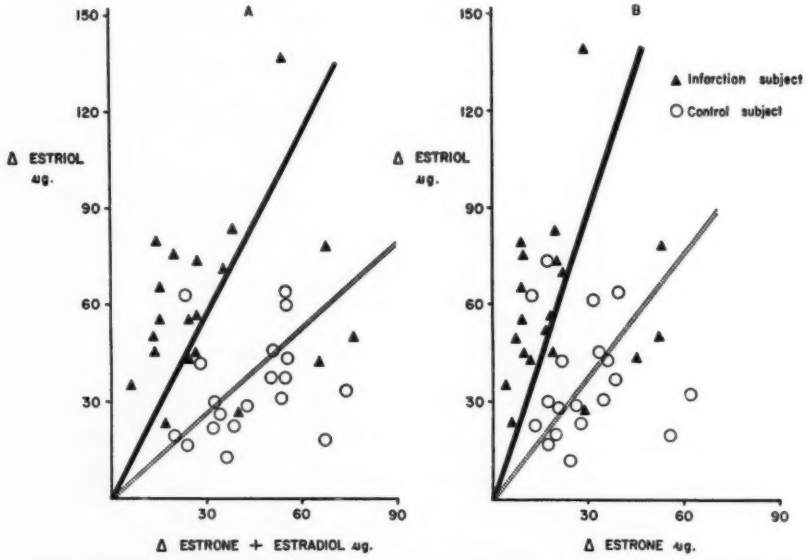

FIG. 2. Relation between increases in urinary excretion of the individual estrogens resulting from injection of estradiol-17β in the subjects of the control and myocardial infarction groups.
A. Relation between increases in urinary excretion of estriol and of estrone + estradiol for the control ○ and the myocardial infarction ▲ groups.
B. Relation between increases in urinary excretion of estriol and of estrone for the control ○ and the myocardial infarction ▲ groups.

Table II lists the values of $\triangle\frac{T}{O+D}$, $\triangle\frac{T}{O}$, and $\triangle\frac{O}{D}$ for the individual subjects together with the percentage of the administered estradiol recovered as estriol, estrone, and estradiol-17β in the urine in the 4 days following injection. Mean and standard error are also shown. For $\triangle\frac{T}{O+D}$, the mean for the

TABLE II

RATIOS OF ESTRIOL, ESTRONE, AND ESTRADIOL EXCRETED IN THE URINE IN RESPONSE TO INJECTION OF ESTRADIOL

Control group					Myocardial infarction group				
Subject	$\Delta \frac{T^*}{O+D}$	$\Delta \frac{T}{O}$	$\Delta \frac{O}{D}$	% recovery	Subject	$\Delta \frac{T}{O+D}$	$\Delta \frac{T}{O}$	$\Delta \frac{O}{D}$	% recovery
IL	0.3	0.3	4.7	17	MAC	0.6	0.9	2.8	29
EG	0.3	0.5	2.2	11	SA	0.6	0.9	2.3	27
RO	0.5	0.5	5.2	21	MCL	0.7	0.9	2.9	15
WA	0.6	0.8	2.6	16	WI	1.1	1.5	3.6	28
ME	0.6	0.9	1.9	20	HA	1.4	3.4	6.5	10
RA	0.7	0.9	2.5	18	DE	1.8	3.8	0.9	17
BE	0.7	0.9	4.2	8	WY	1.9	2.4	4.6	13
YA	0.7	1.1	1.7	17	PAR	1.9	3.2	1.5	27
FO	0.8	1.3	1.5	18	TH	2.0	2.9	2.1	17
MIL	0.8	1.3	1.8	24	SM	2.1	3.0	2.1	15
BA	0.9	1.3	2.1	22	RE	2.2	4.2	1.1	29
WE	0.9	1.4	1.8	15	FR	2.6	3.6	2.7	24
AR	0.9	1.6	1.2	11	CO	2.7	4.9	1.2	44
PO	1.0	1.0	1.0	9	REO	3.4	4.1	4.6	12
WIM	1.1	1.6	2.2	13	MAR	3.4	7.6	1.0	23
SH	1.1	1.9	1.3	30	HO	3.6	6.0	1.3	14
MI	1.2	1.6	2.9	24	PAQ	4.2	7.2	1.4	19
HAN	1.5	2.1	2.7	17	GH	4.6	6.3	2.3	12
CH	2.6	4.1	1.8	23	LA	4.9	9.2	1.1	24
BR	2.8	5.3	1.0	21	ST	5.3	10.5	1.2	8
Mean	1.0	1.5	2.3	18		2.6	4.3	2.4	20
S.E.	0.2	0.3	0.3	1.3		0.3	0.6	0.3	2.0

*For explanation of symbols see Experimental.

'control' group is 1.0 and the mean for the 'myocardial infarction' group is 2.6. The difference between means of the two groups is highly significant ($P<0.001$). For $\triangle\frac{T}{O}$, the mean for the 'control' group is 1.5 and the mean for 'myocardial infarction' group is 4.3. The difference between these means is highly significant ($P<0.001$). For $\triangle\frac{O}{D}$, the mean for the 'control' group of 2.3 is not significantly different from the mean of the 'infarction' group of 2.4. The difference between the mean percentage recovery of the two groups is not significant ($P<0.5>0.1$).

The endogenous excretion of the three estrogens, as determined by analysis of the urine excreted on the day prior to injection, was not significantly different for the two groups. The values have been expressed as ratios of estriol to estrone + estradiol $\left(\frac{T}{O+D}\right)$, estriol to estrone $\left(\frac{T}{O}\right)$, and estrone to estradiol $\left(\frac{O}{D}\right)$ and are listed in Table III.

TABLE III

RATIOS OF ESTRIOL, ESTRONE, AND ESTRADIOL EXCRETED IN THE URINE ON THE DAY PRIOR TO INJECTION OF ESTRADIOL

	Control group			Myocardial infarction group		
	$\frac{T}{O+D}$ *	$\frac{T}{O}$	$\frac{O}{D}$	$\frac{T}{O+D}$	$\frac{T}{O}$	$\frac{O}{D}$
Mean	1.7	3.2	2.5	2.8	4.0	2.3
S.E.	0.3	0.8	0.4	0.5	0.6	0.4
P	0.05	0.10	0.50			

*For explanation of symbols see Experimental.

Fig. 3 shows the consistency of pattern in estrogen excretion following injection of estradiol-17β in a subject at different periods after an acute myocardial infarction. The first experiment was conducted 1 month after infarction when the patient was at bed rest and on anticoagulant therapy. The injection and the urinary estrogen analyses were repeated 1½ months later when the patient was ambulant and at home without anticoagulants. He was then put on a 30 g. fat diet and 2.5 mg. Premarin *R* orally for a 2 month period. Premarin was then discontinued and 5 days later the third experiment was conducted. The relative amounts of the estrogens excreted in response to an intramuscular injection of estradiol-17β were similar in the three periods. The $\triangle\frac{T}{O+D}$ for the three periods was 2.2, 1.8, and 1.9 respectively.

The precision of the analytical method was determined by submitting 464 consecutive duplicate determinations obtained during 18 months of this study to statistical analysis (10). This is shown in Table IV for the three estrogens at different urinary concentrations.

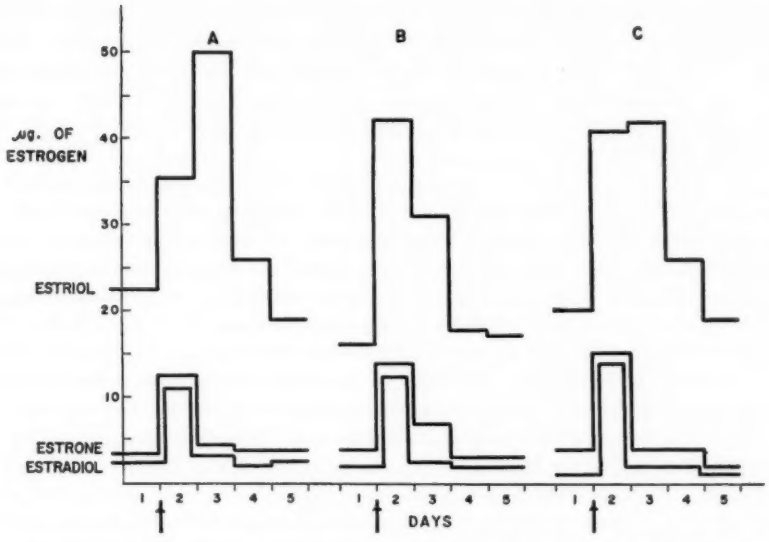

FIG. 3. Urinary excretion of estriol, estrone, and estradiol before and after the injection (↑) of estradiol-17β on three successive occasions in a subject with previous myocardial infarction.

TABLE IV

STANDARD DEVIATION BETWEEN DUPLICATES OF CONSECUTIVE ANALYSES

(μg./24 hours)

Urinary estrogen range (μg./24 hr.)	Estriol	Estrone	Estradiol
0 - 2.5	—	0.38	0.48
2.5 - 5.0	0.22	0.45	0.63
5.0 - 10.0	0.57	0.54	0.74
10.0 - 20.0	1.08	0.88	0.49
20.0 - 40.0	2.09	1.69	—

Discussion

Previous work has established the interrelationships of the three estrogens—estradiol-17β, estrone, estriol—in the human subject. Administration of estradiol-17β causes an increased urinary excretion of estrone (14, 28, 31, 3, 4) and of estriol (28, 3, 4). Administration of estrone causes an increased urinary excretion of estradiol-17β (31, 23) and of estriol (24, 23, 3). On the other hand, administered estriol is not converted into estrone or estradiol-17β (31) but is recovered almost quantitatively from the urine (6). Two of these three findings are supported by *in vitro* studies. Firstly, estradiol-17β and estrone are interconvertible in a variety of human tissue slices (26), in human red blood cells (13), and on perfusion of placenta (16); in fact, a

specific estradiol-17β dehydrogenase has been obtained from placenta (15). Secondly, estriol is not changed on incubation with tissue slices (26). However, many investigators have failed to demonstrate the *in vitro* conversion of estradiol-17β (or estrone) to estriol (15, 25, 26, 27), although this has recently been claimed on the basis of paper chromatographic separation of isotopically-labelled metabolite (9).

The formation of estrone and estriol from estradiol-17β is confirmed in the present investigation which shows that estriol and estrone, as well as urinary estradiol-17β, increase after injection of the latter hormone into male subjects. Moreover, this study establishes the quantitative relationship between the three compounds under these conditions. The proportional increase in urinary estriol to the increase in urinary estrone and estradiol-17β is significantly greater in patients with previous myocardial infarction than in a control group. This indicates a quantitative difference in estrogen metabolism in subjects with and without previous myocardial infarction.

The difference in estrogen metabolism is not a function of age as there is no significant difference between the mean ages of the two groups. Similarly, the time since infarction does not influence the response. As shown in Table I, the period from the episode of myocardial infarction to the beginning of the experiment ranged from 1 week to 2 years. Two cases (DE, MAC) have been studied at repeated intervals without change in the proportions of the three estrogens excreted in response to injection of estradiol-17β. The abnormality of estrogen metabolism seen after myocardial infarction cannot therefore be explained on the basis of age or therapy, nor can it be dismissed as a transient phenomenon of the immediate post-infarction period.

Impairment of liver function also causes an abnormality of estrogen metabolism. This abnormality, however, is not one of increased conversion of estradiol to estriol as seen in this investigation of subjects with previous myocardial infarction. In fact, in cirrhosis of the liver, administered estradiol-17β is largely excreted unchanged, as first shown by Glass, Edmondson, and Soll (11) using bio-assay. These findings were confirmed by Stealy and Stimmel (31) using a chemical method of assay; they showed that the conversion of estradiol to estriol is less in cirrhosis than in the normal subject.

A high level of urinary estriol relative to estrone and estradiol was reported by Diczfalusy and Luft (8) in adrenal cortical carcinoma. In 250 ml. of urine they found 891.5 μg. of estriol, 68.5 μg. of estrone, and 18.4 μg. of estradiol-17β. These authors felt that the high level of estriol was due to the increased production of progesterone demonstrated in this case. This interpretation is in accord with the concepts of Smith and Smith (30). Pearlman, Pearlman, and Rakoff (23), however, were unable to demonstrate any marked difference in estrogen metabolism between men and pregnant women. It is therefore highly improbable that progesterone alters estrogen metabolism. In any event, it is worth noting that increased estrogen production by the adrenal in this one case (8) resulted in an excretion of 10 times as much estriol as estrone and estradiol.

The relative proportions of estriol, estrone, and estradiol-17β excreted in the urine in response to administration of estradiol-17β have been stated by Beer and Gallagher (3, 4) to depend upon the amount administered. These authors reached this conclusion after careful studies with small (0.25 mg.) and large (140–350 mg.) doses of estradiol-17β-16-C^{14} administered to female patients. They found that estriol was the principal urinary excretion product with the small doses and that estrone was the principal urinary excretion product with the large doses. In these experiments, however, the larger doses were given intramuscularly or orally while the small dose, in the subjects whose urine was fractionated, was given intravenously. Possibly the route of administration is important in determining the extent of estrogen catabolism. May and Stimmel (19) found more estrone than estriol in the urine of normal subjects after the ingestion of 2 mg. of estradiol-17β. Similar findings were reported by Brown (7) after the intramuscular injection of 20 mg. of estradiol-17β to a normal subject. These latter findings are consistent with our data on subjects without coronary artery disease.

No significant difference between the 'control' and 'myocardial infarction' group of the present study was found in two indices of estrogen metabolism. Firstly, the mean percentages of administered estrogen appearing in the urine as estriol, estrone, and estradiol during the 4 days after injection do not differ statistically (see Table II). This suggests that the difference between the two groups is not due to alterations in renal function and confirms the conventional tests. Secondly, the relative proportions of estriol, estrone, and estradiol-17β excreted on the day before injection are not significantly different for the two groups (Table III). The difference between the mean $\frac{T}{O+D}$ of the two groups may have been obscured by the lack of precision of the method at values of less than 3 μg./day (see Table IV). On the other hand, it may be that the abnormality in the myocardial infarction group applies only to increased conversion of estradiol-17β to estriol. The hypothesis that a portion of urinary estriol arises by a metabolic pathway other than the conversion of estradiol-17β has been advanced by Marrian (18) and by Brown (5).

This study has demonstrated a significant difference in estradiol-17β metabolism between groups of subjects with and without previous myocardial infarction. Individual cases, however, showed anomalous behavior. Thus, two of our control group (CH, BR) excreted estrogens after the administration of estradiol-17β in the proportions characteristic of the myocardial infarction group (Fig. 2, Table II). Further clinical follow-up is required to determine the significance of increased conversion of estradiol-17β to estriol in these control subjects. On the other hand, present data show that three subjects with previous myocardial infarction metabolize estradiol-17β in a normal manner (Fig. 2). Myocardial infarction can thus occur in subjects not showing an increased conversion of estradiol-17β to estriol. Further study is clearly needed to define the relation of estrogen metabolism to one or more of the pathological mechanisms involved in myocardial infarction.

Acknowledgements

The authors gratefully acknowledge the guidance of Dr. Gilbert Paul, Department of Genetics, McGill University, in the statistical treatment of the results.

The authors are indebted to Drs. M. F. Oliver and J. A. Strong of the Department of Medicine, University of Edinburgh for five cases of myocardial infarction studied in the preliminary stages of this investigation while one of us (W. S. B.) was working in the Department of Biochemistry, University of Edinburgh, under the supervision of Professor G. F. Marrian, F.R.S. We are also pleased to acknowledge the technical assistance of Mrs. A. Alfheim and Miss M. Nilsen.

References

1. ADLERSBERG, D., SCHAEFER, L. E., STEINBERG, A. G., and WANG, C. J. Am. Med. Assoc. **162**, 619 (1956).
2. BAULD, W. S. Biochem. J. (London), **63**, 488 (1956).
3. BEER, C. T. and GALLAGHER, T. F. J. Biol. Chem. **214**, 335 (1955).
4. BEER, C. T. and GALLAGHER, T. F. J. Biol. Chem. **214**, 351 (1955).
5. BROWN, J. B. Lancet, **268**, 320 (1955).
6. BROWN, J. B. Personal communication.
7. BROWN, J. B. Mem. Soc. Endocrinol. **3**, 1 (1955).
8. DICZFALUSY, E. and LUFT, R. Acta Endocrinol. **9**, 327 (1952).
9. DOWBEN, R. M. and RABINOWITZ, J. L. Nature, **178**, 696 (1956).
10. GADDUM, J. H. Pharmacol. Revs. **5**, 111 (1953).
11. GLASS, S. J., EDMONDSON, H. A., and SOLL, S. N. J. Clin. Endocrinol. **4**, 54 (1944).
12. GOFMAN, J. W., JONES, H. B., STRISOWER, B., and TAMPLIN, A. R. Circulation, **14**, 714 (1956).
13. GRAY, C. L. and BISCHOFF, F. Am. J. Physiol. **180**, 279 (1955).
14. HEARD, R. D. H. and HOFFMAN, M. M. J. Biol. Chem. **141**, 329 (1941).
15. LANGER, L. and ENGEL, L. L. Federation Proc. **15**, 966 (1956).
16. LEVITZ, M., CONDON, G. P., and DANCIS, J. Endocrinology, **58**, 376 (1956).
17. LEWIS, L. A., OLMSTEAD, F., PAGE, I. H., LAWRY, E. Y., MANN, G. V., STARE, F. J., HANIG, M., LAUFFER, M. A., and MOORE, F. E. Circulation, **14**, 720 (1956).
18. MARRIAN, G. F. Endeavour, **5**, 35 (1946).
19. MAY, J. A. and STIMMEL, B. F. J. Urol. **59**, 396 (1948).
20. OLIVER, M. F. and BOYD, G. S. Minnesota Med. **38**, 794 (1955).
21. OLIVER, M. F. and BOYD, G. S. Clin. Sci. **12**, 217 (1953).
22. OLIVER, M. F. and BOYD, G. S. Am. Heart J. **47**, 348 (1954).
23. PEARLMAN, W. H., PEARLMAN, M. R. J., and RAKOFF, A. E. J. Biol. Chem. **209**, 803 (1954).
24. PEARLMAN, W. H. and PINCUS, G. J. Biol. Chem. **147**, 379 (1943).
25. PEARLMAN, W. H. and DEMEIO, R. H. J. Biol. Chem. **179**, 1141 (1949).
26. RYAN, K. J. and ENGEL, L. L. Endocrinology, **52**, 287 (1953).
27. RYAN, K. J. and ENGEL, L. L. Endocrinology, **52**, 277 (1953).
28. SCHILLER, J. and PINCUS, G. Arch. Biochem. **2**, 317 (1943).
29. SCHLESINGER, M. J. and ZOLL, P. M. Arch. Pathol. **32**, 178 (1941).
30. SMITH, G. V. S. and SMITH, O. W. Physiol. Revs. **28**, 1 (1948).
31. STEALY, C. L. and STIMMEL, B. F. J. Clin. Endocrinol. **8**, 67 (1948).

THE KINETICS OF AMINO GROUP TRANSFER BY CORN RADICLE TRANSAMINASE[1]

F. S. Cook[2]

Abstract

The kinetics of transamination are complicated by the presence of two substrates whose concentrations change appreciably during the course of the reaction. The only previously published account of the kinetics of this system deviates considerably from classical theory. Equations based on premises of Michaelis and Menten have been shown, however, to accommodate the data on reaction rate in relation to substrate concentration obtained with a corn radicle enzyme preparation by a spectrophotometric method.

Introduction

Transamination involves the transfer of an amino group between two substrates, an amino acid and a keto acid, whose concentrations change appreciably during the course of the reaction. The classical theory of Michaelis and Menten (12), which takes into consideration a change in one substrate only, must be extended, therefore, in order to explain the kinetics of the transaminases and other group-transferring enzymes. A useful extension has envisaged the formation of a ternary complex consisting of the enzyme molecule and two substrate molecules (7, 10, 11). Equations describing the rates of reactions leading to the formation of such a complex have been developed in detail by Laidler and Socquet (9), and by Segal, Kachmar, and Boyer (17).

The only serious attempt to formulate and test kinetic equations for the transaminases has been that of Nisonoff and Barnes (13), who used a pig heart preparation to catalyze the formation of glutamic acid and oxaloacetic acid from aspartic acid and α-ketoglutaric acid. The formulation of these workers is based on the assumption that the enzyme–substrate complexes do not dissociate. It is well known, however, that the glutamic–aspartic transaminase reaction is reversible, and Nisonoff and co-workers themselves have claimed that one pair of substrates competes with the other pair for the same loci on the enzyme (14). If this is the case, reaction *a* and reaction *b* must follow the same pathway, and utilize the same sites on the enzyme surface. The dissociation reactions must, therefore, be appreciable. Furthermore, the kinetic constants formulated by Nisonoff and Barnes apply under limited conditions only, since they are not independent of substrate and enzyme concentrations, and thus do not define the molecular system as does the constant of Michaelis and Menten.

[1] Manuscript received May 27, 1957.
Contribution from the Department of Botany, University of Toronto, Toronto, Ontario. Part of a thesis presented at the University of Toronto in partial fulfillment of the requirements of the degree of Doctor of Philosophy. Part of this study was carried out with financial assistance from the Research Council of Ontario. The spectrophotometer used in this research was provided by a grant from the National Research Council of Canada.
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Kinetic Treatment

An attempt has been made, therefore, to reformulate the relation of reaction rate to substrate concentration for glutamic–aspartic transaminase in closer relation to the Michaelis–Menten theory. A ternary complex is postulated, and the development is compatible with the single displacement theory for group transferring enzymes presented by Koshland (8).

The following assumptions have been made:

1. That the two substrates occupy different sites on the enzyme, and that it is not necessary for one combination to occur before the other substrate can take its place.

2. That the enzyme–substrate complexes dissociate, and that each substrate is free to combine with, and dissociate from its specific locus without being influenced by the other.

3. That the rate of breakdown of the ternary compound to free enzyme and products is small, and does not appreciably affect the equilibrium constants of the other reactions. This assumption was made by Michaelis and Menten (12), but was criticized by Briggs and Haldane (1, 6). If this assumption does not hold K_m is not then a true equilibrium constant, but the analysis of the equations for the single enzyme system is not influenced.

The separate enzyme–substrate combination reactions can then be represented:

$$\begin{aligned} E + A &\rightleftharpoons EA \\ E + B &\rightleftharpoons EB \\ EB + A &\rightleftharpoons EAB \\ EA + B &\rightleftharpoons EAB \\ EAB &\rightarrow E + \text{products} \end{aligned}$$

Whence

$$K_{m_1} = \frac{[E][A]}{[EA]} = K'_{m_1} = \frac{[EB][A]}{[EAB]},$$

$$K_{m_2} = \frac{[E][B]}{[EB]} = K'_{m_2} = \frac{[EA][B]}{[EAB]},$$

and

$$[E] = \frac{K_{m_1}[EA]}{[A]} = \frac{K_{m_2}[EB]}{[B]},$$

$$[EA] = \frac{K_{m_2}[EAB]}{[B]},$$

$$[EB] = \frac{K_{m_1}[EAB]}{[A]}.$$

Therefore

$$[E] = \frac{K_{m_1} K_{m_2}[EAB]}{[A][B]},$$

Let the total enzyme concentration be E_t, i.e.

$$E_t = [EAB] + [EB] + [EA] + [E],$$

$$[1] \qquad E_t = [EAB] + \frac{K_{m_1}[AEB]}{[A]} + \frac{K_{m_2}[EAB]}{[B]} + \frac{K_{m_1} K_{m_2}[EAB]}{[A][B]}.$$

The final step in the reaction sequence is the breakdown of the ternary compound,

$$EAB \xrightarrow{k} E + \text{products}.$$

The measured velocity is

$$v = k\,[EAB].$$

Then, by rearranging equation [1],

$$k\,[EAB] = \frac{k\,E_t\,[A][B]}{[A][B] + K_{m_2}\,[A] + K_{m_1}\,[B] + K_{m_1}\,K_{m_2}}$$

and

$$[2] \qquad \frac{1}{v} = \frac{1}{V_x} + \frac{K_{m_2}}{V_x\,[B]} + \frac{K_{m_1}}{V_x\,[A]} + \frac{K_{m_1}\,K_{m_2}}{V_x\,[A][B]},$$

where

$$V_x = kE_t.$$

If the concentration of one substrate, [A], is varied while the other, [B], is held constant, equation [2] may be written

$$[3] \qquad \frac{1}{v} = \left\{\frac{[B] + K_{m_2}}{V_x\,[B]}\right\} + \frac{1}{[A]}\left\{\frac{K_{m_1}\,([B] + K_{m_2})}{V_x\,[B]}\right\}.$$

This is the equation of a straight line. The ordinate intercept is the reciprocal of the maximum velocity, V_{m_1}, and can be seen to be dependent upon the level at which [B] is held constant, on the concentration of the enzyme, E_t, on the velocity constant, k, and on the dissociation constant of the B-enzyme complex, K_{m_2}. If the slope of the straight line is divided by the ordinate intercept, the value for K_{m_1} is obtained.

A straight line is obtained if [B] is varied while [A] is held constant,

$$[4] \qquad \frac{1}{v} = \left\{\frac{[A] + K_{m_1}}{V_x\,[A]}\right\} + \frac{1}{[B]}\left\{\frac{K_{m_2}\,([A] + K_{m_1})}{V_x\,[A]}\right\},$$

and values for the maximum velocity, V_{m_2}, and for K_{m_2} can be calculated.

If the concentrations of both substrates are varied and kept equal,

$$[A] = [B] = [S],$$

$$[5] \qquad \frac{1}{v} = \frac{1}{V_x} + \frac{K_{m_2}}{V_x\,[S]} + \frac{K_{m_1}}{V_x\,[S]} + \frac{K_{m_1}\,K_{m_2}}{V_x\,[S]^2}.$$

It is evident that, in this case, the relation between $1/v$ and $1/[S]$ is not linear. Equation [5] can be written in another form,

$$\frac{1}{v} = \frac{1}{V_x}\left\{\frac{[S]^2 + K_{m_2}[S] + K_{m_1}[S] + K_{m_1}\,K_{m_2}}{[S]^2}\right\}$$

and

$$[6] \qquad v = V_x\left\{\frac{[S]^2}{([S] + K_{m_1})\,([S] + K_{m_2})}\right\}.$$

When only one substrate is varied, a plot of its concentration against the reaction velocities results in a rectangular hyperbola, but equation [6] describes a sigmoidal curve. The shape of the sigmoid depends upon the relative values of K_{m_1} and K_{m_2}.

Equation [6] can be written in a more general form to fit cases where the concentrations of the two substrates are not equal,

$$[7] \qquad v = V_x \left\{ \frac{[A]}{[A] + K_{m_1}} \times \frac{[B]}{[B] + K_{m_2}} \right\}.$$

This form of the equation is identical with equation (1) of Connell (3), derived to describe transpeptidase kinetics.

Theoretical curves fitting equation [7] are plotted in Fig. 6. The values of V_x, K_{m_1}, and K_{m_2} are read from Table II, and their derivation is described below. Two of the curves are typical rectangular hyperbolae. When both substrates are varied simultaneously, and their concentrations kept equal, however, the curve is sigmoidal. This is only slightly evident at very low substrate concentrations. At high concentrations this curve rises above the hyperbolae approaching a higher maximum velocity. These results are discussed further and compared with experimental data below.

The experimental work was confined to a study of the glutamic–aspartic transaminase prepared from radicles of corn seedlings. In all cases the reaction was carried out as designated by *b* in the following:

$$\text{glutamic acid} + \text{oxaloacetic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \text{aspartic acid} + \alpha\text{-ketoglutaric acid.}$$

Materials and Methods

Source of Enzyme

Homogenates prepared from the terminal centimeter of the radicles of 3-day-old maize seedlings (*Zea mays* L. var. Golden Rocket) were used in all experiments described. The seeds were sterilized and germinated, and the radicles excised as described in a previous paper (4).

Recently excised radicle segments were homogenized with cold 0.05 *M* phosphate buffer of pH 8.0 in a glass homogenizer of the Potter–Elvehjem type (18). The tube of the homogenizer was kept immersed in ice-water during this operation. The structural parts of the tissue were tossed down by a 5 minute centrifugation at 2000 r.p.m. Only a small fraction of the total nitrogen and transaminase activity is lost in this centrifugate. The cloudy, yellow-brown supernatant was then centrifuged at 15,000 r.p.m. in a Phywe Pironette centrifuge. This threw down a small, yellow pellet, and left a clear, yellow supernatant in which most of the transaminase remained. Repeated washings of the pellet with buffer would remove much of what transaminase activity was thrown down.

The supernatant was dialyzed for 48 hours against running tap water which varied in temperature from 7° C. to 11° C. Two glass beads were placed in the cellophane sac, and it was continuously inverted for the entire period of dialysis. This treatment caused the formation of a slight precipitate, which was discarded by a 5 minute centrifugation at 3500 r.p.m. This precipitate was shown to contain no transaminase activity. The clear, light yellow supernatant was used as the source of the glutamic–aspartic transaminase in all experiments.

A single enzyme preparation was used to obtain all the data on the effects of varying the concentrations of substrates. These experiments were repeated with the same results using a second preparation.

Analytical Procedure

Accurate kinetic data, dependent upon initial velocities of reactions, were obtained by an adaptation of the spectrophotometric method of Nisonoff *et al.* (13, 15). The use of changes in optical density of a system brought about by the appearance or disappearance of certain substrates to measure transamination activity was first suggested by Green *et al.* (5), and has been utilized also by Cammarata and Cohen (2). The increase in optical density is measured as oxaloacetic acid is produced when aspartic acid and α-ketoglutaric acid are added to a suitable enzyme preparation. Readings can be accurately made at 15-second intervals during the first 2 or 3 minutes of a reaction, and straight lines giving initial velocities can be accurately drawn (Fig. 1). The first reading was made at 30 seconds from zero time in order to allow for mixing.

Optical densities were measured at a wavelength of 280 mμ, and the optical absorption coefficients of the various substrates and products at this wavelength are given in Table I. All obey Beer's Law within the range of concentrations used in these experiments. Since oxaloacetic acid spontaneously decomposes in solution the optical density at a given concentration was measured by dissolving an accurately weighed quantity of powdered oxaloacetic acid in the buffer solution at zero time, mixing the substances well to dissolve the acid, making a series of readings at measured time intervals, and extrapolating to zero time.

TABLE I

OPTICAL ABSORPTION COEFFICIENTS OF GLUTAMIC–ASPARTIC TRANSAMINASE SUBSTRATES AT λ 280 mμ

Substrate	Optical absorption coefficient, optical density unit per μM. per ml.
Oxaloacetate	0.657
α-Ketoglutarate	0.0525
Aspartate	0.0006
Glutamate	0.0010

Concentration range of oxaloacetate: 0 to 3 μM. per ml.
Concentration range of other substrates: 0 to 20 μM. per ml.
All substrates dissolved in 0.05 *M* phosphate buffer, pH 8.0.

Nisonoff *et al.* (15) have shown that in their systems a given concentration of any one of the components contributes to the optical density of a mixture of the components to the same degree as when the concentration of each component is measured alone. This was verified with the substrates used in these studies.

Optical density readings were made with a Beckman model DU spectrophotometer, and standard ultraviolet accessory equipment. Constant temperature was maintained with water circulated from a constant temperature bath through four thermospacers, two placed on either side of the cell compartment. Solutions within the cell were maintained at $38 \pm 0.2°$ C. The reactions were carried out within the silica absorption cells, the volume in all cases being maintained at 3.0 ml.

The procedure adopted was to add 0.05 *M* phosphate buffer of pH 8.0, 0.5 ml. of the enzyme preparation, and one substrate (that required in the greater amount) to the absorption cell, and to incubate the mixture for 3 minutes. At zero time the other substrate was added, the cell tipped at least six times, quickly replaced in the cell compartment, and the first reading made at 30 seconds. The silica cell and all components were heated to 38° C. before an experiment was begun.

Reagents

The amino and keto acids used in these studies were purchased from Nutritional Biochemicals Corporation. L-Aspartic acid and α-ketoglutaric acid served as substrates for the reactions studied. It was necessary to use oxaloacetic acid and L-glutamic acid only to obtain their optical absorption coefficients at 280 mμ, and to verify the additive properties of the optical density values of the components of the transaminase system.

Results

Properties of the Enzyme Preparation

A difficulty encountered in the spectrophotometric method was the high absorption of the corn radicle enzyme preparation at 280 mμ. The rigorous dialysis against cold running tap water eliminated a great deal of the absorbing components without changing the transaminase activity.

Aside from the breakdown of oxaloacetic acid, no evidence came to light of side reactions involving components of the transaminase system. It has been shown (4) by the paper chromatogram method that when the reaction was stopped at zero time by phosphoric acid and sodium tungstate, when α-ketoglutaric acid was omitted from the system, or when the enzyme preparation was heated in a boiling water bath for 10 minutes, all of the aspartic acid could be recovered and no other ninhydrin-reacting substances appeared. When transamination was allowed to proceed, the glutamic acid formed plus the remaining aspartic acid accounted for 96% of the moles of amino acid added as aspartic acid. When aspartic acid was omitted from the otherwise complete reaction mixture using either frozen radicles or a homogenate as the enzyme source, no ninhydrin-reacting substances appeared on the paper.

In the spectrophotometer, when any one component was left out, the optical density of the mixture remained at a value equivalent to the sum of the optical densities of the other components. Thus, the paper chromatogram

experiments show that amino acids are neither destroyed nor synthesized when the transaminase system is incomplete. The spectrophotometric experiments indicate that α-ketoglutaric acid remains unchanged, since this compound has an absorption coefficient high enough at 280 mμ (Table I) to enable one to detect changes of the order of 0.02 μM./ml. These results indicated that it was unnecessary to take special precautions, such as employing anaerobic conditions or purifying the enzyme preparation to a greater degree, in order to obtain reliable measurements of transaminase activity.

Time Course of the Reaction

Representative curves showing the time course of the reaction at four different substrate concentrations are shown in Fig. 1. Since the spectrophotometric method measures changes in the concentration of oxaloacetic acid, it is necessary that initial velocities be determined. The spontaneous decom-

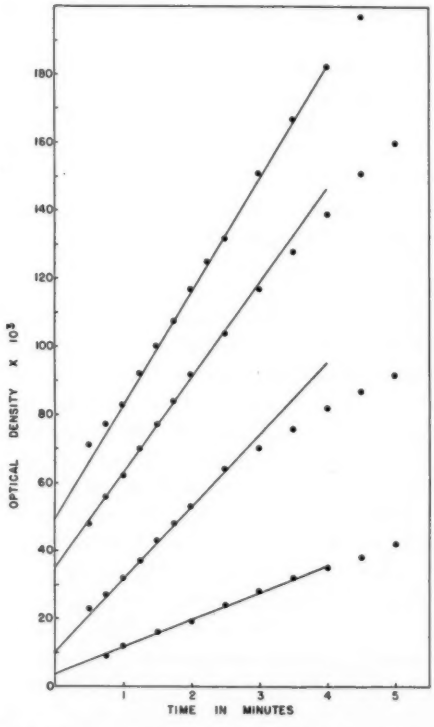


FIG. 1. Typical time course curves of the glutamic–aspartic transaminase reaction *b* at four different concentrations of substrates. Tangents show initial velocities. λ 280 mμ. System and conditions of reactions: 0.5 ml. enzyme preparation; varying volumes of 0.030 *M* L-aspartic acid; varying volumes of 0.030 *M* α-ketoglutaric acid; 0.05 *M* phosphate buffer, pH 8.0, to make final volume of 3.0 ml.; temp. 38° C. Enzyme preparation: corn radicle tips, 1.0 cm. in length, homogenized in phosphate buffer, centrifuged at 20,000 *g*, and dialyzed.

position of this product adds substantially to the effect of the other rate-reducing factors such as the activity of the reverse reaction, decrease in substrate concentration, and deterioration of the enzyme with time. From Fig. 1 it will be seen that the initial rate begins to fall off between 2 and 4 minutes from zero time.

In a few cases a slight lag is observed at the beginning of the measured reaction; this may be seen in two of the curves of Fig. 1. From the appearance of their graphs it is evident that Nisonoff and Barnes (13) observed the same phenomenon, and at temperatures below 27° C. they found the lag to be considerable. Such a lag might be attributable to problems of molecular orientation, which enzymic catalysis must involve. In a system in which two ionizable substrates take part the proceedings in the vicinity of the active centers must be complex, and the time required for a steady velocity to be reached can be expected to be finite. At 38° C. the lag, if present, is small and when allowed for does not diminish the accuracy with which the initial velocity is measured.

Velocities in concentration units per minute can be obtained from measurements made in optical density units per minute by means of the absorption coefficients of Table I (13). The production of 1 μM. per ml. each of oxaloacetic acid and glutamic acid causes an increase in optical density equal to

$$(0.657+0.0010) - (0.525+0.0006) = 0.605.$$

Therefore, the velocity in optical density units per minute divided by 0.605 gives the velocity in μM. per ml. per minute.

Michaelis–Menten Constants

The curves obtained when the initial velocities are plotted against substrate concentration are shown in Figs, 2, 3, and 4. In Fig. 2 α-ketoglutaric acid concentration is varied while aspartic acid concentration is held constant at 5.0 μM. per ml. In Fig. 3 aspartic acid is varied while α-ketoglutaric acid is held at 5.0 μM. per ml. In Fig. 4 the concentrations of both substrates are varied and kept equal to each other. The curves of Figs. 2 and 3 are rectangular hyperbolae and are very similar to each other, indicating that the K_m values are similar. K_{m_1} and K_{m_2} are calculated by substituting experimental values for v and [A] in equation [3] and for v and [B] in equation [4], respectively, and employing the method of least mean squares (Table II).

The pig heart enzyme of Nisonoff and Barnes gives a much steeper initial slope when α-ketoglutaric acid alone is varied than when only aspartic acid is varied. This indicates a higher affinity for the keto acid by the animal enzyme, whereas in the corn enzyme the affinities for the two substrates are more nearly equal. For the corn enzyme $K_{m_1} = 0.93$ μM. per ml., and $K_{m_2} = 1.33$ μM. per ml. Nisonoff and Barnes do not give corresponding values, but by applying the Michaelis–Menten theory to their data, they can be determined approximately from their graphs: $K_{m_1} = 6$ μM. per ml. and $K_{m_2} = 0\,8$ μm. per ml. Panalaks (16) obtained values more nearly equal with soybean enzyme: $K_{m_1} = 2.2$ μM. per ml. and $K_{m_2} = 2.8$ μM. per ml.

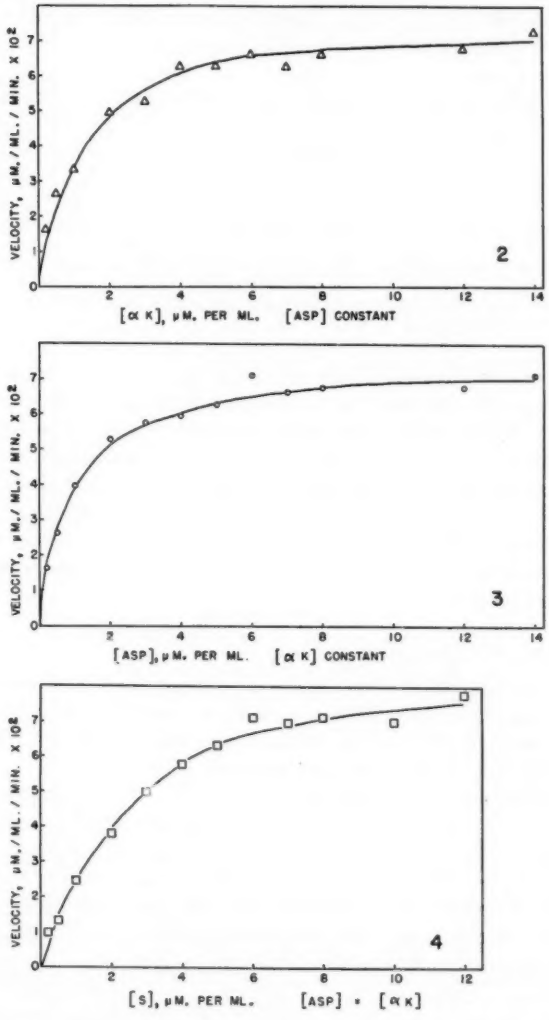


FIG. 2. Effect of varying the concentration of α-ketoglutaric acid on the initial velocity of the glutamic–aspartic transaminase reaction *b*. The concentration of L-aspartic acid is held constant at 5.0 μM. per ml. System and enzyme preparation as in Fig. 1.

FIG. 3. Effect of varying the concentration of L-aspartic acid on the initial velocity of the glutamic–aspartic transaminase reaction *b*. The concentration of α-ketoglutaric acid is held constant at 5.0 μM. per ml. System and enzyme preparation as in Fig. 1.

FIG. 4. Effect of varying the concentrations of both substrates simultaneously on the initial velocity of the glutamic–aspartic transaminase reaction *b*. The concentrations are held equal to one another. System and enzyme preparation as in Fig. 1.

TABLE II

KINETIC CONSTANTS OF GLUTAMIC–ASPARTIC TRANSAMINASE OF CORN RADICLES

Experiment	V_{m_1}, μM./ml./min.	V_{m_2}, μM./ml./min.	V_x, μM./ml./min.	K_{m_1}, μM./ml.	K_{m_2}, μM./ml.
[A] Varied*	0.075	—	—	0.93	—
[B] Varied†	—	0.080	—	—	1.33
[A] = [B]‡	—	—	0.091	—	—

* Concentration of aspartic acid varied; concentration of α-ketoglutaric acid held constant at 5.0 μM./ml.

† Concentration of α-ketoglutaric acid varied; concentration of aspartic acid held constant at 5.0 μM./ml.

‡ Concentrations of both substrates kept equal, and varied simultaneously.

System and conditions of reactions as in Fig. 1.

These latter values are higher than those for the corn enzyme, but this might be attributed to the fact that Panalaks did not measure initial velocities, but only degree of change after a 10 minute reaction period. The only suggestion offered by Nisonoff and Barnes for the great difference in rate of access of the two substrates in their systems was that the ratio of concentration of active species to total concentration may be very different for the two. This seems doubtful in view of the results with corn and soybean. Whatever it is that causes this peculiar behavior with the animal enzyme seems to be absent from the plant preparations.

Maximum Initial Velocities

The curve of Fig. 4 has a shallow initial slope, and rises above the other two curves approaching a higher maximum velocity, as predicted. This may be seen more readily in Fig. 5, where all three curves of Figs. 2, 3, and 4 are plotted together without the experimental points.

Table II gives the values for the maximum reaction velocities of these three curves. The values of V_{m_1} and V_{m_2} are obtained from equations [3] and [4], respectively, in the same manner as the K_m values are derived. The value for V_x is estimated by substituting in equation [6] for K_{m_1} and K_{m_2}, chosen values for substrate concentrations, [S], and corresponding observed initial reaction velocities, v. The value of V_x given in Table II is the average of those obtained using 12 experimental values of v (mean of the differences equals 8%).

Comparison of Observed and Theoretical Values of v

The theoretical curves of Fig. 6 are obtained by substituting the V_x, V_m, and K_m values of Table II in equation [7]. A comparison of Fig. 5 with Fig. 6 shows that the three curves drawn visually through the experimental points bear the same general relationship to one another as do the three theoretical curves. That the agreement between the theoretical and observed values is close can be seen by plotting the curves of Fig. 6 along with the observed reaction velocities. Such a comparison has been shown only for

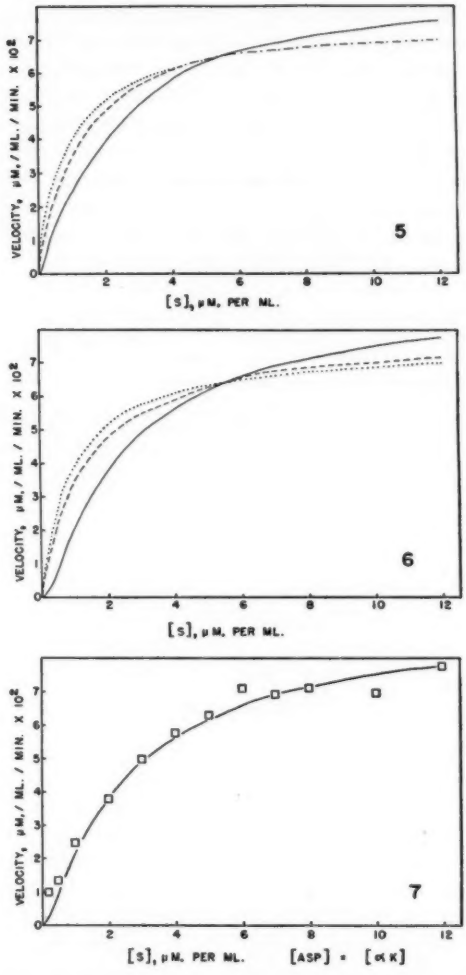


FIG. 5. Effect of varying the concentrations of the substrates on the initial velocity of the glutamic–aspartic transaminase reaction *b*. The data of Figs. 2, 3, and 4 are plotted together. System and enzyme preparation as in Fig. 1.
Dotted line: L-aspartic acid only varied.
Dashed line: α-ketoglutaric acid only varied.
Solid line: Both substrates varied simultaneously.

FIG. 6. Theoretical curves derived from equation [7] showing relation between substrate concentration and initial velocity.
Dotted line: L-aspartic acid only varied.
Dashed line: α-ketoglutaric acid only varied.
Solid line: Both substrates varied simultaneously.

FIG. 7. A comparison of theoretical values of initial velocities with observed values; L-aspartic acid and α-ketoglutaric acid concentrations kept equal and varied simultaneously. The theoretical curve of Fig. 6 (solid line) is plotted with the experimental points of Fig. 4.

the case where the concentrations of both substrates are varied and kept equal (Fig. 7), but the agreement is equally close in the other two cases. A more complete comparison of 32 measured velocities with corresponding theoretical values is made in Table III. The mean of the relative differences of these values is 8%. The largest discrepancies are found at low substrate concentrations particularly in the experiment where both substrates are varied (Fig. 7), and where initial velocities are relatively very low. These discrepancies can be accounted for by the experimental errors involved in measuring the low rates of change of optical density, and in pipetting into the cells volumes of substrate solutions of the order of 0.01 ml.

TABLE III

COMPARISON OF OBSERVED VELOCITIES OF GLUTAMIC–ASPARTIC TRANSAMINASE REACTIONS WITH VELOCITIES CALCULATED BY MEANS OF EQUATION [7]

Concentration of aspartic acid, μM. per ml.	Concentration of α-ketoglutaric acid, μM. per ml.	Calculated velocity, μM. per ml. per min. $\times 10^2$	Observed velocity, μM. per ml. per min. $\times 10^2$
0.2	5.0	1.33	1.64
0.5	5.0	2.63	2.62
1.0	5.0	3.90	3.97
2.0	5.0	5.14	5.29
3.0	5.0	5.75	5.77
4.0	5.0	6.12	5.95
5.0	5.0	6.35	6.28
6.0	5.0	6.52	7.11
8.0	5.0	6.74	6.76
12.0	5.0	7.01	6.78
5.0	0.2	1.04	1.63
5.0	0.5	2.17	2.64
5.0	1.0	3.41	3.32
5.0	2.0	4.78	4.95
5.0	3.0	5.51	5.26
5.0	4.0	5.97	6.26
5.0	5.0	6.28	6.28
5.0	6.0	6.51	6.63
5.0	7.0	6.68	6.28
5.0	8.0	6.82	6.62
5.0	12.0	7.17	6.78
0.5	0.5	0.88	1.30
1.0	1.0	2.05	2.48
2.0	2.0	3.80	3.79
3.0	3.0	4.89	4.96
4.0	4.0	5.62	5.79
5.0	5.0	6.16	6.28
6.0	6.0	6.55	7.11
7.0	7.0	6.84	6.96
8.0	8.0	7.10	7.12
10.0	10.0	7.49	6.95
12.0	12.0	7.76	7.74

System and conditions of reactions as in Fig. 1.

Another test of agreement between our hypotheses and the data is the relationship between V_x and V_m. From equation [3] it is seen that when [B] is held constant at 5.0 μM. per ml.,

$$V_{m_1} = \frac{V_x\,[B]}{[B] + K_{m_2}}. \qquad [8]$$

From equation [4], when [A] is held at 5.0 μM. per ml.,

$$V_{m_2} = \frac{V_x\,[A]}{[A] + K_{m_1}}. \qquad [9]$$

Now V_{m_1} and V_{m_2} can be calculated by substituting the values given in Table II for V_x, K_{m_2}, and K_{m_1}. These calculated values are compared with the observed values in Table IV.

TABLE IV

A COMPARISON OF OBSERVED AND CALCULATED VALUES OF V_{m_1} AND V_{m_2}

	Calculated values, μM./ml./min.	Observed values, μM./ml./min.	Relative difference, %
V_{m_1}	0.072	0.075	5
V_{m_2}	0.078	0.080	2

Calculated from equations [8] and [9].
System and conditions of reactions as in Fig. 1.

Discussion

It is evident that our data fit the derived equations as closely as the experimental error allows. The equations have been developed from premises that are essentially those of Michaelis and Menten, and it is clear, therefore, that the classical theory can accommodate the existing data on transaminase kinetics. It is difficult, however, to establish superiority of the present formulation merely by comparing curves. It might be possible to derive a number of families of equations which fit the data to an approximation, but unless the basic assumptions are more reasonable than those of the classical theory, and unless the equations provide constants with unambiguous significance for the theory of enzyme action, such a formulation could not replace classical theory.

It is for such reasons that the formulation given here is presented in preference to that of Nisonoff and Barnes. These authors have made assumptions some of which are dubious, and at least one of which is inconsistent with their own later conclusions. The constants of their equations are complex, apply under limited conditions only, and are of doubtful meaning for enzymic reactions in general.

How narrow the formal differences between the two formulations may be is made clear by examining equation [5]. The factor $K_{m_1} K_{m_2}/V_x\,[S]^2$, under

the conditions prevailing with the corn radicle enzyme, is rather small, and if we neglect this term equation [5] becomes

$$[10] \qquad \frac{1}{v} = \frac{1}{V_x} + \frac{1}{S}\left\{\frac{K_{m_1} + K_{m_2}}{V_x}\right\}.$$

This is a straight line equation, and a plot of v against [S], therefore, is a rectangular hyperbola. The ordinate intercept of the straight line gives $1/V_x$, and the slope gives $(K_{m_1} + K_{m_2})/V_x$. The slope divided by the ordinate intercept is equal to $K_{m_1} + K_{m_2}$. The sum of these two values read from Table II is equal to 2.26 μM. per ml. The substrate concentration giving $\frac{1}{2}V_x$ can be read from Fig. 4, and is equal to 2.53 μM. per ml. The percentage difference between these two values is about 12, a greater discrepancy than that found between the theoretical and observed values for V_m using equation [7] (Table IV).

Again, according to the Nisonoff and Barnes equations, the slope of the line representing the double reciprocal plot when both substrates are varied should equal the sum of the slopes of the two lines obtained when each substrate is varied alone. These authors give the values of only two of their slopes, however, and it is impossible to test this relationship using the data which they present. Furthermore, it follows from this relationship that the curve obtained by varying both substrates cannot possibly coincide with either of the other two. Actually, although it will not be sigmoidal, it will resemble the curve of Fig. 7 in that it will have a less steep initial slope than either of the curves representing the variation of only one substrate, and it will rise towards a higher maximum velocity than those. Nisonoff and Barnes, however, show this curve as coincident with that obtained when aspartic acid alone is varied. This discrepancy casts further doubt on the assumptions which permit them to reach the conclusion that when both substrates are varied together the relation of reaction velocity to substrate concentration is hyperbolic as it is when the substrates are varied separately.

Acknowledgments

The author is indebted to Professor G. H. Duff for advice and assistance throughout these studies, for his careful criticism and suggestions during the preparation of the manuscript, and for obtaining financial assistance. Thanks are due also to Professor C. S. Hanes of the Department of Biochemistry, University of Toronto, for the privilege of consultation, and for making available unpublished results of recent work in his laboratory.

The author wishes also to thank several others: Professor D. F. Forward for many helpful suggestions, Mrs. N. Nolan for her careful drawing of the figures, Mrs. J. Moffat for preparing some of the plant material, and the people of Canada for financial help through the Department of Veterans' Affairs.

References

1. BRIGGS, G. E. and HALDANE, J. B. S. Biochem. J. **19**, 338 (1925).
2. CAMMARATA, P. S. and COHEN, P. P. J. Biol. Chem. **193**, 45 (1951).
3. CONNELL, G. E. Ph. D. Thesis, University of Toronto, Toronto, Ont. 1955.
4. COOK, F. S. Can. J. Biochem. and Physiol. **34**, 15 (1956).
5. GREEN, D. E., LELOIR, L. F., and NOCITO, V. J. Biol. Chem. **161**, 559 (1945).
6. HALDANE, J. B. S. Enzymes. Monographs on biochemistry. Longmans, Green & Co., Ltd., London. 1930.
7. KALCKAR, H. M. *In* Currents in biochemistry. *Edited by* D. E. Green. Interscience Publishers, Inc., New York. 1946.
8. KOSHLAND, D. E. *In* The mechanism of enzyme action. *Edited by* W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md. 1954.
9. LAIDLER, K. J. and SOCQUET, I. M. J. Phys. & Colloid Chem. **54**, 519 (1950).
10. LUVALLE, J. E. and GODDARD, D. R. Quart. Rev. Biol. **23**, 197 (1948).
11. MICHAELIS, L. *In* Currents in biochemistry. *Edited by* D. E. Green. Interscience Publishers, Inc., New York. 1946.
12. MICHAELIS, L. and MENTEN, M. L. Biochem. Z. **49**, 333 (1913).
13. NISONOFF, A. and BARNES, F. W., JR. J. Biol. Chem. **199**, 713 (1952).
14. NISONOFF, A., BARNES, F. W., JR., and ENNS, T. J. Biol. Chem. **204**, 957 (1953).
15. NISONOFF, A., HENRY, S. S., and BARNES, F. W., JR. J. Biol. Chem. **199**, 699 (1952).
16. PANALAKS, T. Ph.D. Thesis, University of Toronto, Toronto, Ont. 1956.
17. SEGAL, H. L., KACHMAR, J. F., and BOYER, P. D. Enzymologia, **15**, 187 (1952).
18. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. Manometric techniques and related methods for the study of tissue metabolism. Burgess Publishing Company, Minneapolis, Minn. 1945.

ON DISTRIBUTION AND INHERITANCE OF ATYPICAL FORMS OF HUMAN SERUM CHOLINESTERASE, AS INDICATED BY DIBUCAINE NUMBERS[1]

W. KALOW AND N. STARON

Abstract

Type and activity of serum cholinesterase (pseudocholinesterase) have been studied in nearly 1700 human sera. The type was determined by an inhibition test with dibucaine; per cent inhibition achieved under specified conditions was termed dibucaine number (DN). The DNs can be classified into the three groups—usual, intermediate, and atypical. This triple division is consistent with the assumption that there exist two different types of enzyme which occur either in pure form or in a mixture; the proportion of the two enzymes in the mixture varies considerably from person to person but there are equal proportions on the average. Most observations on the inheritance of DN can be explained by the presence of two autosomal allelic genes without dominance, each gene causing the formation of one of the two types of enzyme. Some observations pointed to additional factors which may be variants of the normal gene, or modifying genes which influence the expressivity of the normal gene. There was an excess of male offspring in the investigated families but a connection with DN not established. In a healthy population, the frequency of the atypical gene was calculated to be 0.0140 ± 0.0036. Thus roughly one out of 30 persons has an intermediate DN but only one out of 3000 to 10,000 is expected to have an atypical DN.

Dibucaine (Cinchocaine, TN Nupercaine, Percaine, Perkain) is known as a local anaesthetic and inhibitor of human serum cholinesterase (pseudocholinesterase). The dibucaine number (DN) (3) is a measure of the degree of inhibition of serum cholinesterase obtained with dibucaine under standardized conditions, and expressed as a percentage. The technique of determining DN has been described in detail (3); it consists essentially of measurements by ultraviolet spectrophotometry of the esterase activity of diluted human serum, the measurements being performed with benzoylcholine as substrate in the absence and in the presence of dibucaine.

An unusual DN is part of an unusual pattern of substrate specificities and inhibition characteristics of human serum cholinesterase. There is reason to believe that different values of DN indicate structural differences between the active sites of otherwise identical or very similar enzyme proteins. The detailed data will be given in a subsequent publication. So far, it has been reported that the DN of a given individual remains constant, even if the level of esterase activity changes (3). Patients with low DN react in a predictable fashion with "prolonged apnoea" to the muscular relaxant succinylcholine (5).

[1]Manuscript received July 23, 1957.
Contribution from the Department of Pharmacology, University of Toronto, Toronto, Ontario.

Experimental Procedures

The Subjects of Investigation

All subjects could be classified as belonging to either an arbitrarily defined population, to a small, special group, or to a family.

1. The population was composed as follows:

(*a*) 194 students, 88% of them males. All volunteered for the test.

(*b*) 346 laborers, about 85% of them males. The skills in this group varied widely. Blood samples were taken routinely at the start of employment.

(*c*) 205 patients in the Toronto General Hospital, about 50% of them males. The sera were obtained without selection from the Wassermann Laboratory of the Hospital.

(*d*) 811 patients[2] of two different mental hospitals, about 50% of them males. They consisted of several groups: one group of 324 was selected by random sampling from the hospital files; one group of 265 was made up of patients recently admitted to one of the hospitals; one group of 68 was composed of candidates for electroshock treatment; and the last group of 154 had been selected for a clinical investigation which had no connection with this study of cholinesterase.

The population thus consisted of 540 healthy persons and 1016 patients, a total of 1556 subjects. Since there was no statistically significant difference in the occurrence of atypical DNs and esterase levels between the patients and the healthy persons, all their data have been combined for the purpose of studying the frequency distribution of DNs.

2. The special group. The investigation of these subjects was requested by their physicians because of a prolonged apnoea after an injection of succinylcholine (5).

3. The families. Seven families were examined. The first person of a family to be investigated will be referred to as the proband of that family. Five of the probands were students with intermediate DNs, while the two probands with low DN belonged to the above-mentioned special group. Most members of the investigated families were residing in the southern part of the Province of Ontario; all these, and those living in the Province of Quebec and in the States of Michigan and New York, were visited personally by one of us (N.S.). A few samples were received by mail, mainly from Northern Ontario and Alberta.

Laboratory Methods

Samples of blood were taken from a cubital vein and allowed to clot. Serum was removed for investigation.

The DN was determined by ultraviolet spectrophotometry. The substrate was 5×10^{-5} *M* benzoylcholine, the concentration of dibucaine 10^{-5} *M*, and the dilution of serum 1:100. Solvent was phosphate buffer of pH 7.4 at

[2]A recently published brief account (2) mentioned the investigation of 1400 mentally ill patients. Most of these patients were tested under circumstances which permitted detection of the cases with low DN but missed the intermediate ones. Hence, these data were not included in the present paper.

about 25° C. The measurements were made with a Beckman Recording Spectrophotometer at 240 mμ. Details of this procedure have been reported (3).

As a by-product of the determination of DN, data on esterase levels have been obtained. The results were expressed in micromoles of acetylcholine hydrolyzed per hour by 1 ml. of serum at 37° C. The calculation of these units from the optical data has been described (6). The physical and enzymological basis of the spectrophotometric method has been formulated in detail (4).

While the esterase activity of a sample of blood or serum decreased when it was left at room temperature, the DN remained unaffected as long as it could be measured. Hence, whenever there was a reason to suspect the inadvertently prolonged exposure to warm temperature of samples during their transportation, the DNs were reported but the esterase levels were omitted. This accounts for a slight discrepancy between the sizes of the two sets of data in this report.

Experimental Error

The standard deviation of the method of determining esterase activity was about ±10 esterase units. The corresponding deviation of the inhibition test was slightly above ±2 DN.[3] These figures refer to data obtained on different days. For experiments performed on the same day, these error terms were smaller. They were about 1.5 DN with the routine technique and about 0.6 DN if especially careful procedures were employed. In sera with low esterase activity, the error of determination of DN tended to be larger than in the usual samples.

In the samples from families, all DNs were determined at least in duplicate. In the population, all measurements which resulted in DNs below 72 and above 84 were repeated. All others were reinvestigated only on occasion, either to assess the magnitude of experimental error, or whenever there was reason to suspect a faulty technique. However, towards the end of this investigation, DNs between 72 and 73 were also rechecked routinely. The fact that part of the samples were investigated twice and another part once did not essentially alter the frequency distribution which will be reported below.

Results

Distribution in a Population

The frequency distribution of DNs in the population is shown in Fig. 1. The majority of DNs are between 85 and 71. Another accumulation of values, although small by comparison, lies between 66 and 58. Below 58

[3]After completion of the studies it was noticed that one of the ionization constants of dibucaine was roughly 7.5 in terms of pK_a. Hence, a variation of ±2 DN can be caused by a variation of ±0.12 pH units. During this work, the pH of 6-liter batches of fresh buffer solutions was adjusted to be between 7.38 and 7.40 at room temperature. By more frequent controls of pH, the experimental error could have been reduced.

the incidence of DNs decreases. Altogether there are 59 DNs between 70 and 43, there are none between 43 and 20, but two at 19 and 12 respectively.

This arrangement of DNs does not conform to one normal frequency distribution. The simplest description of the data can be given in terms of three groups, which will be referred to as (1) high, usual, or typical; (2) intermediate; (3) low, rare, or atypical. The dividing lines between the groups are chosen at 70 and 20. The high group is described very nearly by a mean of 78.75 ± 0.06 and a standard deviation (SD) of ± 2.28. The mean of the intermediate group is 61.9 ± 0.3, with a SD of 3.3. The two low values of DN are around 16.

The division of human sera according to DN is not visible if measurements are confined to the conventional determination of esterase activities.

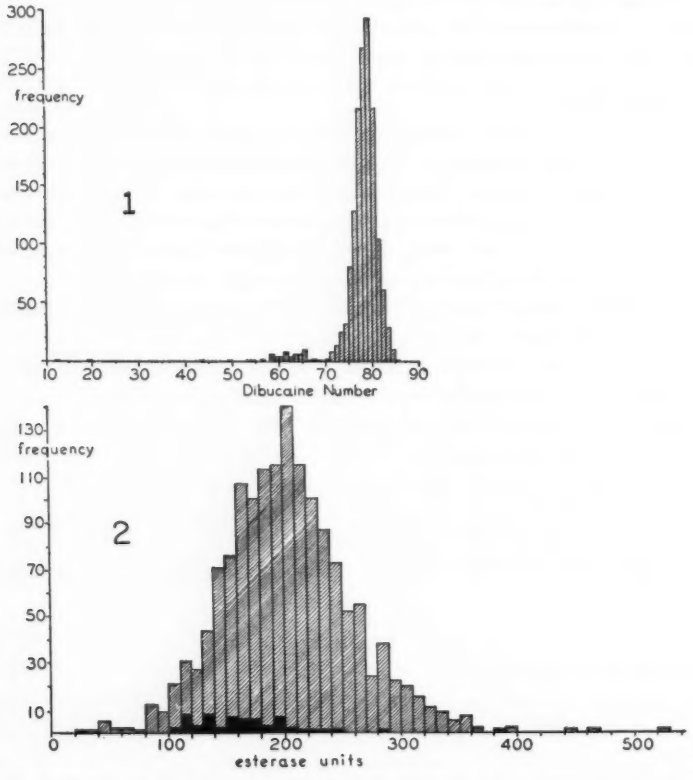


FIG. 1. Frequency distribution of DNs in an arbitrarily defined population consisting of 1556 laborers, students, and patients.

FIG. 2. Frequency distribution of esterase levels in an arbitrarily defined population. Determinations from essentially the same sera as the data shown in Fig. 1. The black columns indicate the esterase levels of sera with DN below 70.

This is shown by the frequency diagram (Fig. 2) of esterase levels of essentially the same people whose DNs were presented in Fig. 1. The mean is 203.7 ± 1.6, the SD 61.1. The esterase levels of persons with DN below 70 are given in black. Although their average esterase activity is below the general mean, for any individual with a given DN the esterase activity can vary widely (cf. 3).

The experimental error of the screening tests is large enough to account for the variation of high DN which is shown in Fig. 1. Nevertheless, differences between high DNs were suggested by a set of repetitive tests made within a few hours, with care to achieve greater accuracy than during the ordinary routine test. That is, when the DNs of four persons were each determined five times in quick succession, the individual averages ranged from 77.7 to 79.5. According to an analysis of variance, this difference between persons was significant on the 5% level. This difference expressed as SD was here ±1.6 DN; it was 1.2 DN in a test previously described (3) and ±1.7 DN in a third, independent, series.

Differences between DNs classified as intermediate exceeded distinctly the variation due to the experimental error of the routine test. This can be seen from previously presented examples (3). Further illustrations will be given below.

Although only two sera with DN below 20 have been found by the regular screening method (Fig. 1), nine more such sera have become known in other ways; four were from patients who reacted to succinylcholine with grossly prolonged apnoea (5); three were from relatives of persons with low DN (see below); and one serum was that of an interested colleague. The average DN of all these sera was 15.8±0.7, the SD was ±2.8. The experimental error was relatively large and it was not possible to demonstrate differences from person to person between these DNs, perhaps because the sera were not available at the same time.

Of the 11 sera with low DNs, 10 were found in males (aged between 3 and 76) and only one in a woman. The probability is less than 1 in 50 that this difference from an even distribution between sexes is accidental. However. there is no further suggestion of a connection between sex and DN in these data. Intermediate DNs have nearly the same average in both sexes, and the proportion of males is the same in the high and the intermediate groups.

Both of the sera with low DN that were found by the regular screening method occurred among the mentally ill patients. Hence, the data of Fig. 1 do not necessarily represent the frequency of low DN in the healthy population. The part of the sample that consisted of 540 healthy laborers and students contained only 15 intermediate DNs, i.e. 2.8%. Among the 1016 patients were 44 intermediate DNs, i.e. 4.3%. This difference is not statistically significant but one cannot yet exclude the possibility of a genetic relationship between decreased DNs and certain types of illness, particularly mental illness. This is still being investigated.

Distribution in Several Families

The frequency distribution of DNs among relatives of persons with intermediate or low DNs is shown in Fig. 3. The classification of DNs into three groups is more distinct than in the corresponding plot of the population (Fig. 1). Relative to the high group, the intermediate and low groups are large. They constitute 36.3% of the sample in contrast to 3.9% of the population. If measurements are confined to determinations of esterase activity, the classification of sera is again not apparent (Fig. 4, cf. Fig. 2).

Table I gives the results of an investigation of parents and sibs of five students with intermediate DNs. In each case, either father or mother also had an intermediate DN. Family 2 shows a peculiarity: there are twins who have indistinguishable DNs of intermediate magnitude. Also, the DNs of father and first son seem to be similar and intermediate but they are significantly different from the DNs of the twins as well as from that of the mother. These distinctions are supported by an analysis of variance of repetitive tests on the sera of this family.

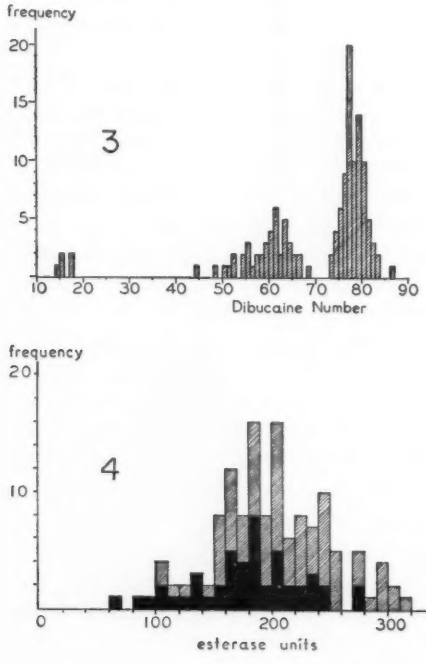


FIG. 3. Frequency distribution of DNs among 135 members of seven unrelated families.

FIG. 4. Frequency distribution of esterase levels among members of seven unrelated families. Determinations from essentially the same sera as the data shown in Fig. 3. The black columns show the esterase levels of sera with DN below 70.

TABLE I

TYPE AND ACTIVITY OF SERUM CHOLINESTERASE IN FIVE FAMILIES

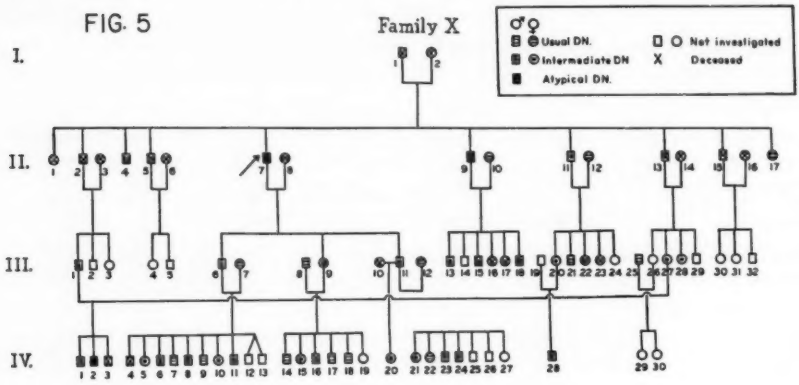
	Family No.	Father	Mother	Sons			Daughter
				1	2	3	
Type	1	Int.	Typ.	Int.	Int.	Int.	*Int.*
DN		61.5	83.2	59.1	56.5	57.4	61.3
Units		189	218	175	134	167	114
Type	2	Int.	Typ.	*Int.*	Int.*	Int.*	
DN		64.0	77.9	65.8	44.7	48.1	
Units		210	230	198	129	131	
Type	3	Typ.	Int.	*Int.*			
DN		79.8	55.0	60.4			
Units		204	184	245			
Type	4	Typ.	Int.	*Int.*			
DN		80.5	68.3	64.5			
Units		237	198	162			
Type	5	Int.	Typ.				*Int.*
DN		63.0	80.6				60.0
Units		233	180				110

Int. = intermediate DN.
Typ. = typical DN.
Italics = the probands.
*Identical twins, as established in the Hospital for Sick Children, Toronto, by Dr. Norma Ford Walker.

The data on two extensively investigated families are shown in detail in the Appendix, and they are compiled in geneological trees (Figs. 5 and 6). The probands of both families were found because they reacted to succinylcholine with prolonged apnoea. In the figures, the probands are indicated by arrows. The blood of the proband of family *Y*, his wife, and his daughter have been tested for the groups ABO, MNS, CDE (Rh factor), *K* (Kell), *JK* (Kidd), *FY* (Duffy), *Le* (Lewis), and *Lu* (Lutheran).[4] The distribution of their blood groups was in agreement with the paternity indicated in Fig. 6. The blood groups of the son with low DN could not be investigated; he was 3 years of age and unco-operative and so no second blood sample was obtained. The father-in-law of the proband of family *Y* had an intermediate DN, his mother-in-law a high DN. The data from these and other in-laws are not shown in the figures.

In family *X* as well as in family *Y*, the triple division of DN was very distinct. However, the averages of the intermediate groups differed between the families (Fig. 7). That is, the average intermediate DN in family *X* was 60.8 ± 0.8, while the average intermediate DN in family *Y* was 54.1 ± 1.1. This difference is statistically significantly ($P < 0.01$). On the other h nd, the means of the high groups in the two families were nearly identical and so were the means of the low groups.

[4]We are grateful to Dr. Bruce Chown, Children's Hospital, Winnipeg, for these determinations.



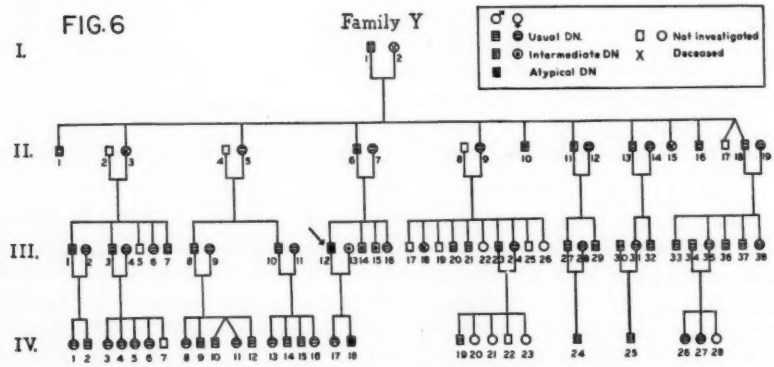


FIG. 5. Pedigree of family *X* in which atypical DN occur. The proband is indicated by the arrow. Data on each individual are given in the Appendix.

FIG. 6. Pedigree of family *Y* in which atypical DN occur. The arrow points to the proband. Data on each individual are given in the Appendix.

The esterase activity of the proband of family *Y* was 67 ± 1 units, that of his son was 104 ± 2 units. In family *X*, the three sera with low DN had esterase activities of 80, 90, and 132 units, respectively.

Analysis and Discussion

The division of DNs into three groups is consistent with kinetic data to be published which suggest that sera with high DN contain one type of cholinesterase, with low DN another type of cholinesterase, and with intermediate DN a mixture of these two enzymes. The high and the low groups are more uniform than the intermediate group; that is, with the routine technique it was not possible to demonstrate differences between DNs classified as high or between those classified as low, while differences between

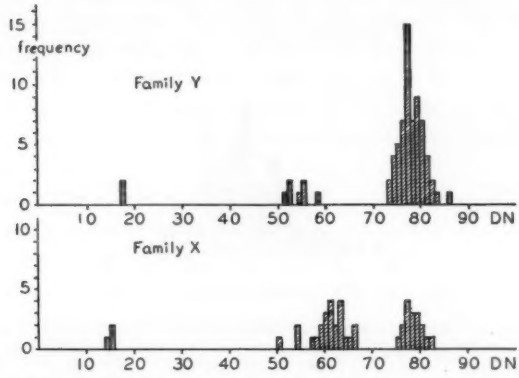


FIG. 7. Frequency distributions of DN in the two largest families investigated (cf. Figs. 5 and 6). Note the positions of the intermediate groups; the difference of their means is significant on the 1% level.

intermediate DNs were readily apparent (cf. 3). Only by special effort was it possible to obtain some evidence for the existence of real, though small, differences between high DNs.

The intermediate DNs not only show the greatest variation from person to person but their distribution curve is skew, as indicated by the extended tail to the left (Figs. 1 and 3), and their position is asymmetrical between the high and the low groups. Theory demands that such characteristics—large scatter, skewness, and asymmetrical position—should be produced if there is a mixture of enzymes in which the proportion of typical esterase varies from person to person. This theory is based on the following arithmetic which has been verified experimentally by mixing sera with high and low DNs. The typical serum has usually about 210 units of esterase activity without inhibitor and 44.5 units with inhibitor, the DN thus being $100-(100\times44.5/210)=78.8$. The atypical serum has usually about 80 units of esterase activity without inhibitor, and 67.2 units with inhibitor, the DN thus being 15.8. Then, a mixture containing equal parts of both sera must have a DN of $100-[100\times(44.5+67.2)/(210+80)]=61.5$. A mixture containing 66.6% of typical and 33.3% of atypical esterase would have a DN of $100-[100\times(89+67.2)/(420+80)]=68.8$. By such calculations, one can translate each intermediate DN into a "percentage of typical esterase". The mean of this percentage of typical esterase is 50.2 ± 0.9, the SD being 9.0. These statistics are expressed by the smooth curve drawn in Fig. 8. The fit of the data to this curve is imperfect. While the discrepancies are not significant ($P > 0.05$), attention during future research will be directed to the possibility of the existence of discrete subclasses within the intermediate group.

Overwhelming evidence for a familial occurrence of low and intermediate DN is given by the contrast between Figs. 1 and 3. This familial occurrence cannot be due to surroundings but must be due to inheritance; for instance,

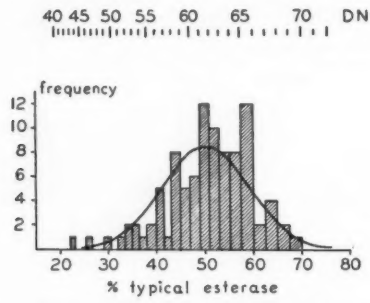


FIG. 8. Frequency distribution of intermediate DNs on a theoretical scale. All intermediate DNs from the investigated population and from the families are combined in this figure; the data from the twins (Table I) are averaged. The scale of the abscissa is calculated under the assumption that sera with intermediate DN contain a mixture of atypical and typical esterase. The smooth curve indicates the theoretical frequency distribution which should be obtained if the average mixture consists of 50% typical esterase, the SD being ±9%.

sibs had more often a similar DN than spouses (Figs. 5 and 6, Table I), although very few of the adult sibs lived together.

Many types of gene interactions could produce a threefold expression of an inherited feature (9). However, the simplest genetic situation which can lead to three phenotypes is the existence of two allelic autosomal genes without dominance. Whether the familial distribution of DN could be explained by this mechanism can be tested as follows: Let the two genes be symbolized by the letters *A* and *B*. Then, persons with high DN have two typical genes for cholinesterase, which may be designated as *AA*. Low DN would be due to two atypical genes *BB*, while the intermediate groups would possess one of each allele, symbolized by *AB*. Between three such genotypes, six types of mating are possible, of which four, perhaps five, have been observed.

In family *X* (Fig. 5) there are two marriages of the type *AA* and *BB* (II 7 and 8, II 9 and 10), which should produce nothing but intermediate (*AB*) offspring. That is the case (III 6, 9, 11 and 13, 18). There are four marriages of the type *AA* and *AB* (II 11 and 12, III 6 and 7, 8 and 9, 11 and 12). The expected offspring should be of two types in equal proportions, namely typical and intermediate. The observed offspring consisted of 11 intermediate and 9 high DNs. There is one marriage between first cousins (III 1 and 27), both of whom have intermediate DN. This represents mating type *AB* and *AB*, with expected offspring of all three types, *AA*, *AB*, and *BB* in the ratio 1:2:1. Of the two living sons, one is intermediate, the other atypical, representing the genotypes *AB* and *BB*.

The contents of Table I may be taken as five further examples of mating types *AA* and *AB*. Of 10 offspring, two are identical twins. Excluding a co-twin and the five probands, one may figure with four opportunities for an independent ascertainment of DN. In all four instances, the DNs were intermediate. The difference between observation and expectation is not significant.

Ten marriages of the type AA and AA are recorded for family Y (Fig. 6). One would expect all the offspring to be of type AA. This is the case, since all 27 children investigated from these marriages had a high DN.

The assumption of two autosomal alleles could explain completely all the data up to this point. The following observations require additional concepts for explanation:

1. The variation from person to person within the group of high DN.
2. The difference of intermediate DN between father and identical twins in family 2, Table I.
3. The low average value of intermediate DN in family Y.
4. The low DN of the proband of family Y.

All of these observations fit the idea that there exist different "normal" genes, $A_1, A_2, \ldots, A_n$.

Differences between normal genes could obviously explain the person-to-person variations between typical DN. There is, however, not yet any direct evidence for a genetic determination of the variants. Considering family 2 of Table I, the mother might have a set of genes A_1A_2. Then, the eldest brother could be of a genotype A_1B, while the twin brothers are A_2B. In family Y there might be a gene A_3 present which causes in the combination A_3B the relatively low values of the intermediate DNs (Fig. 7). There might further exist a gene A_4 which tends to be completely suppressed. Thus the proband (III 12) of family Y could be assumed to have the gene combination A_4B, his DN being low because only the atypical gene is effective. If this were true, his mother (II 7) would have to have a set of genes A_3A_4. Since A_4 is ineffective and A_3 only moderately effective, she should have a low esterase activity but a normal DN; that is the case (esterase units 103, DN 79).[5] This observation gains considerable weight since the esterase activity of her daughter (III 16) is very similar (units 102.5, DN 77.3). Also, the esterase activity of the proband's daughter (IV 17) should be low; it is lower (159 units) than average but not so low as that of her aunt or grandmother. Furthermore, if the proband has only gene B causing the formation of enzyme, while his son (IV 18) has two genes (BB) which are both effective, the son should have a higher esterase activity than the father. That is the case; the units are 104 and 67 respectively.

Instead of the differences between normal genes $A_1, A_2, \ldots, A_n$, one could assume a single type of gene, A, the expressivity of which is influenced by modifying genes. Both these hypotheses could explain most observations equally well but not the variation between typical DNs. How the latter variation could be caused by modifying genes is difficult to visualize. However both these hypotheses are not mutually exclusive; jointly they might come nearest to the truth.

[5]She had a cholecystectomy 2 years prior to this investigation but has been in good health ever since. There was no reason to assume a hidden liver damage capable of producing a low esterase activity but clinical tests could not be performed.

In families *X* and *Y* there are more male than female offspring, the numbers being 40 vs. 26 in family *X* and 41 vs. 28 in family *Y*. This is not likely to have occurred by chance ($P < 0.05$); however, the unusual sex ratio has no obvious connection with DN, since there are only eight unusual DNs in family *Y*. The proportion of intermediate DNs within each sex is about equal. The same observation has been made for the population. Hence, the excess of males (10 out of 11) among the persons with low DN remains hard to explain. It is certain that the average esterase activity of males in the population exceeds that of females by 20 units ($P < 0.001$, unpublished data).

Lehman and Ryan (8) have collected over several years from many hospitals the sera of patients who reacted with prolonged apnoea to the muscular relaxant succinylcholine. In this way, they found 13 patients with low serum cholinesterase activity[6] in the absence of liver damage. Of these patients, 7 were females and 6 were males (7). The same authors investigated 11 relatives of two of these patients and found familial occurrence of low serum cholinesterase activity. Another family of six, with inherited low esterase activity, was described by Allott and Thompson (1). In this case, the proband had a low, his wife a high, and the three sons an intermediate esterase activity.[7] The sixth person was the proband's brother, also with intermediate activity. All these observations fit into the genetic pattern presented here in terms of two autosomal alleles. If all of the 13 patients observed by Lehman and Ryan, and the proband of Allott and Thompson, have low DN, the total number of such persons known is 25, of which 17 are males and 8 females. Such a deviation from an equal sex distribution could have occurred by chance (*P* between 0.1 and 0.2). In other words, the combined data do not support the evidence for a sex influence upon the occurrence of atypical esterase.

Since there is no indication that a substantial number of atypical genes might have escaped detection, one may estimate the frequency of the atypical gene. As mentioned above, there is the possibility of an increased occurrence of intermediate and low DNs among some patients. Hence the estimate is better based on the observations in healthy persons. There were 15 intermediate DNs among 540 students and laborers. That is, the frequency of the gene was 0.0140 ± 0.0036. That means, the incidence of atypical homozygotes with low DN should be 1 in 5100, the limits being 1:3370 and 1:10,500. This agrees with the observations of Lehmann and Ryan (8), who found the frequency of their cases with familial low esterase activity "well below 1:1000".

The literature contains one other example of a genetically determined esterase. Sawin and Glick (10) studied the hydrolysis of atropine by an esterase which occurs in the blood serum of some rabbits. The occurrence of the enzyme is inherited through a gene (A_s) borne in the same chromosome as the gene (*E*) for extension of black pigment in the coat. The A_s gene is incompletely dominant, homozygotes producing the enzyme more effectively

[6]Their esterase units, multiplied by three, equal the units employed here.
[7]Their esterase units, multiplied by two, equal the units employed here.

than heterozygotes. The enzyme is absent at birth and appears in the serum at the age of 1 month. It is found in higher concentrations in females, and in a larger proportion of females than of males.

Note Added in Proof

Intermediate DNs have been found in some samples of umbilical cord blood. Hence, the type of human serum cholinesterase is established before birth.

Two women with low DN have been found recently. Hence, of all 13 persons known to have low DN, three are females and 10 are males. This deviation from an equal distribution between sexes is not significant.

Acknowledgments

Our warm thanks are due to all who helped to make this work possible. Dr. Norma Ford Walker, Hospital for Sick Children, Toronto, guided the planning and the evaluation of the genetic studies. Prof. D. B. W. Reid, Department of Epidemiology and Biometrics, gave generously of his time for statistical advice. It is impossible to mention by name all those who have helped with the collection of samples; of particular importance were the efforts of Drs. D. R. Gunn, S. Jackson, and B. McPherson. All those who permitted samples of their blood to be taken deserve special thanks. Encouragement and useful advice were given to us by Professors J. K. W. Ferguson, H. Cullumbine, and B. F. Crocker, and other members of the Departments of Pharmacology and Biochemistry. The technical work was performed by K. Genest and Mrs. M. Slubicki.

Financial assistance was received from a Mental Health Grant, Department of Health, Province of Ontario.

References

1. ALLOTT, E. N. and THOMPSON, T. C. Lancet, **II**, 517 (1956).
2. KALOW, W. Lancet, **II**, 576 (1956).
3. KALOW, W. and GENEST, K. Can. J. Biochem. and Physiol. **35**, 339 (1957).
4. KALOW, W., GENEST, K., and STARON, N. Can. J. Biochem. and Physiol. **34**, 637 (1956).
5. KALOW, W. and GUNN, D. R. J. Pharmacol. Exptl. Therap. **120**, 203 (1957).
6. KALOW, W. and LINDSAY, H. A. Can. J. Biochem. and Physiol. **33**, 568 (1955).
7. LEHMANN, H. Private communication.
8. LEHMANN, H. and RYAN, E. Lancet, **II**, 124 (1956).
9. SNYDER, L. H. The principles of heredity. 4th ed. D. C. Heath & Company, Boston. 1951.
10. SAWIN, P. B. and GLICK, D. Proc. Natl. Acad. Sci. U.S. **29**, 55 (1943).

NOTE: An Appendix follows.

APPENDIX

FAMILY *X* (cf. FIG. 5)

Generation	Individuals	Esterase units	DN	Remarks
II	7	81	15.7	Mentally ill
	8	272	78.6	
	9	91	15.3	
	10	291	80.4	
	11	163	65.2	
	12	270	77.9	
	13	187	66.3	
	17	225	82.0	
III	1	233	61.4	
	6	203	63.9	
	7	171	81.2	
	8	298	76.6	
	9	178	66.5	
	11	202	62.7	
	12	186	77.7	
	13	172	54.9	
	18	155	57.4	
	20	165	63.0	
	21	202	79.4	
	22	189	78.0	
	23	203	78.8	
	25	320	79.2	
	27	108	59.5	
	28	197	62.8	
IV	1	228	61.8	
	2	132	14.0	
	5	186	55.4	
	6	177	60.4	
	7	247	76.4	
	8	247	64.8	
	9	308	77.2	
	10	228	59.7	
	11	273	58.6	
	14	258	75.4	
	15	186	54.6	
	16	201	50.3	
	17	257	77.3	
	18	210	78.8	
	20	231	63.0	
	21	186	60.6	
	22	250	79.7	
	23	209	61.5	
	24	215	61.2	
	28	273	63.4	

FAMILY *Y* (cf. FIG. 6)

Generation	Individuals	Esterase units	DN	Remarks
II	1	171	74.0	
	5	106	81.2	
	6	151	52.2	
	7	103	79.0	
	9	242	75.5	
	10	—	56.8	Mentally ill
	11	183	58.8	
	13	155	77.0	
	14	235	78.8	
	16	191	79.0	
	—	215	78.0	Wife of 16
	18	209	76.2	
	19	230	78.5	
III	1	210	79.0	
	2	235	81.5	
	3	290	80.2	
	4	125	77.3	
	6	201	80.3	
	7	240	86.6	Mongolian idiot
	8	205	74.3	
	9	207	80.7	
	10	186	80.6	
	11	161	77.2	
	12	67	17.8	Proband
	13	179	54.3	
	—	164	55.5	Father of 13
	—	307	76.2	Mother of 13
	14	181	52.0	
	15	146	51.0	
	—	157	78.0	Wife of 15
	16	102	77.3	
	20	186	77.9	
	21	180	74.7	
	—	142	76.7	Wife of 21
	23	195	76.5	
	24	160	73.1	
	27	150	81.8	
	28	207	75.5	
	29	—	77.8	Mongolian idiot
	30	184	77.8	
	31	161	77.7	
	32	207	78.0	
	—	155	76.0	Wife of 32
	33	276	75.7	
	34	190	75.3	
	35	179	82.4	
	36	248	79.3	
	37	248	77.0	
	38	204	75.7	

FAMILY *Y* (cf. FIG. 6)—*Concluded*

Generation	Individuals	Esterase units	DN	Remarks
IV	1	189	82.2	
	2	201	74.2	
	3	151	83.2	
	4	166	80.1	
	5	224	77.8	
	6	258	78.8	
	8	220	79.7	
	9	194	81.4	
	10	162	78.8	
	11	167	73.3	
	12	294	80.9	
	13	222	76.6	
	14	219	77.0	
	15	245	79.0	
	16	240	80.7	
	17	159	79.9	
	18	104	17.9	
	19	168	76.5	
	24	—	79.9	
	25	195	77.1	
	26	223	79.2	
	27	245	78.4	

CONTENTS OF VOLUME 35

CORRECTION

Volume 35, page 633. Along the ordinate of Figure 1 the figures 40, 45, 50, etc., should read 4.0, 4.5, 5.0, etc.

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An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

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References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should not be given and initial page numbers only are required. The names of periodicals should be abbreviated in the form given in the most recent *List of Periodicals Abstracted by Chemical Abstracts.* All citations should be checked with the original articles, and each one referred to in the text by the key number.

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All figures (including each figure of the plates) should be numbered consecutively from 1 up, in arabic numerals, and each figure should be referred to in the text. The author's name, title of the paper, and figure number should be written in the lower left-hand corner of the sheets on which the illustrations appear. Captions should not be written on the illustrations (see Manuscripts (i)).

(ii) ***Line drawings***

Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used unless it is desired to have all the co-ordinate lines show. All lines should be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots should be solid black circles large enough to be reduced if necessary. Letters and numerals should be neatly made, preferably with a stencil (**do NOT use typewriting**), and be of such size that the smallest lettering will be not less than 1 mm. high when reproduced in a cut 3 in. wide.

Many drawings are made too large; originals should not be more than 2 or 3 times the size of the desired reproduction. In large drawings or groups of drawings the ratio of height to width should conform to that of a journal page but the height should be adjusted to make allowance for the caption.

The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.

(iii) ***Photographs***

Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard with no space or only a **very** small space (less than 1 mm.) between them. In mounting, full use of the space available should be made (to reduce the number of cuts required) and the ratio of height to width should correspond to that of a journal page ($4\frac{3}{4} \times 7\frac{1}{2}$ in.); however, allowance must be made for the captions. Photographs or groups of photographs should not be more than 2 or 3 times the size of the desired reproduction.

Photographs are to be submitted in duplicate; if they are to be reproduced in groups one set should be mounted, the duplicate set unmounted.

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A total of 50 reprints of each paper, without covers, are supplied free. Additional reprints, with or without covers, may be purchased.

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Any reprints required in addition to those requested on the author's reprint requisition form must be ordered officially as soon as the paper has been accepted for publication.

Contents